Receptor-mediated endocytosis of insect lipoprotein: insight into LDL receptor functioning

Receptor-gemedieerde endocytose van insecten-lipoproteïne: inzicht in het functioneren van de LDL receptor

(met een samenvatting in het Nederlands)

PROEFSCHRIFT

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With a giant leap, the locust takes off to engage flight;
powerful strokes of its wings ensure it remains airborne
for up to 10 hours without pause.

With a pathetic jump it barely leaves ground;
not even the feverish flapping of its arms
can prevent the human to plummet like a brick.

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Voor mijn ouders.

CONTENTS

ABBREVIATIONS

apoB:	apolipoproteinB
apoB-100:	apolipoprotein B-100
apoLp:	apolipophorin
CHO:	Chinese hamster ovary
CR:	cysteine-rich repeat
DAG:	diacylglycerol
EGF:	epidermal growth factor
ERC:	endocytic recycling compartment
FFA:	free fatty acid
FH:	familial hypercholesterolemia
HDL:	high-density lipoprotein
HDLp:	high-density lipophorin
HMW:	high molecular weight
IDL:	intermediate-density lipoprotein
IF:	immunofluorescence
L_4:	fourth instar
L_5:	fifth instar
LDL:	low-density lipoprotein
LDLp:	low-density lipophorin
LDLR:	low-density lipoprotein receptor
LMW:	low molecular weight
Lp:	lipophorin
LPL:	lipoprotein lipase
LpR:	lipophorin receptor
LRP:	low-density lipoprotein receptor-related protein
LT:	LysoTracker Yellow
OG:	Oregon Green 488
PA:PAI-1:	plasminogen activator-plasminogen activator inhibitor-1
RAP:	receptor-associated protein
RRE:	receptor recycling efficiency
TAG:	triacylglycerol
Tf:	transferrin
TfR:	transferrin receptor
TMR:	tetramethylrhodamine
VLDL:	very-low-density lipoprotein
VLDLR:	very-low-density lipoprotein receptor
Vg:	vitellogenin
VgR:	vitellogenin receptor
wt:	wild-type

CHAPTER 1

General introduction: lipoproteins and lipoprotein receptors

Based partly on:

Dick J. Van der Horst, D. Van Hoof, Wil J.A. Van Marrewijk and Kees W. Rodenburg

Alternative lipid mobilization: The insect shuttle system

Mol Cell Biochem **(2002) 239:** 113-119

Apolipoprotein-facilitated lipid transport: lipoproteins

Animal organisms accumulate lipids in body depots that function as fuel stores. These lipids are usually stored in the form of triacylglycerol (TAG), the fatty acids of which are derived from lipids that are either absorbed from the food or resulting from *de novo* synthesis (Bernlohr et al., 2002). Generally, the transport of hydrophobic lipids from intestine to target tissues through the aqueous circulation involves specific carrier proteins, namely apolipoproteins (Frayn, 1996). A large variety of apolipoproteins have been identified and characterized in mammals. Lipoproteins are lipid-apolipoprotein complexes, composed of lipids associated with a single or multiple apolipoproteins (Jonas, 2002).

Mammals make use of a wide array of lipoproteins that display different compositions and functions (Frayn, 1996; Ganong, 2001; Jonas, 2002; Vance, 2002); the nomenclature of these particles is based on centrifugational density. Lipids derived from dietary intake are incorporated into chylomicrons produced by the intestinal enterocytes (Fig. 1; Fielding and Fielding, 2002). These huge lipid-protein complexes (∅ 750-10000 Å) with a density of less than 0.94 g/ml are composed of a single non-exchangeable apolipoprotein (apo)B-48, accompanied by several copies of apoA, apoC and apoE as the major exchangeable apolipoproteins, and TAG as the main lipid moiety. Chylomicrons are particularly transported to adipose tissue (Fielding and Fielding, 2002), where lipids are extracellularly hydrolyzed by lipoprotein lipase (LPL). The free fatty acids (FFAs) released are taken up by the adipocytes, and resynthesized to TAG. During this process of selective lipid unloading, the exchangeable

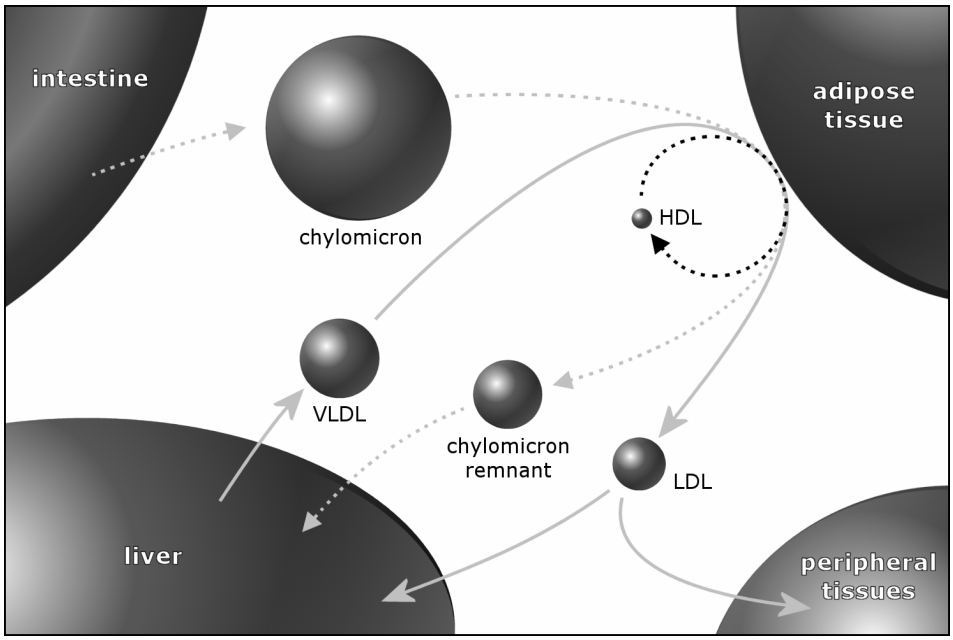

Figure 1. Schematic overview of lipoprotein transport in mammals. Chylomicrons and VLDL are produced by the intestine and liver, respectively. Both lipoproteins are converted to particles with a higher density upon extracellular LPL-mediated lipid extraction at the adipose tissue. During this process, HDL functions as the acceptor for the relieved exchangeable apolipoproteins. The liver takes up chylomicron remnants and LDL, the latter is also taken up by peripheral tissues.

apolipoproteins detach from the lipoprotein and associate with another lipoprotein, high-density lipoprotein (HDL) with a density of 1.063-1.21 g/ml (∅ 75-100 Å). The resulting chylomicron remnant, harboring apoB-48, acquires additional cholesteryl esters from HDL-mediated transfer and is transported to the liver where it is taken up and degraded (Fielding and Fielding, 2002). The liver produces very low-density lipoprotein (VLDL), a particle comprising a single copy of the non-exchangeable apoB-100, and multiple exchangeable apolipoproteins, including apoE (for review see Shelness and Sellers, 2001). VLDL has a density of 0.94-1.006 g/ml (∅ 300-800 Å) and contains mainly TAG in addition to cholesterol, cholesteryl esters, and phospholipids (Jonas, 2002). Similar to chylomicrons, VLDL is unloaded extracellularly; LPL mediates hydrolysis of the majority of its TAG cargo, while the resulting FFAs are taken up by adipose tissue and resynthesized to TAG (Bernlohr et al., 2002). Lipid transfer from VLDL to adipose tissue occurs in conjunction with the VLDL receptor (VLDLR; for review see Tacken et al., 2001; Schneider, 2002), a process during which HDL functions as the acceptor for the relieved exchangeable apolipoproteins. The resulting particle is an intermediate-density lipoprotein (IDL), which is further converted into low-density lipoprotein (LDL) with a density of 1.006-1.063 g/ml (∅ 200-220 Å). Through the action of the plasma enzyme lecithin-cholesterol acyltransferase, cholesteryl esters are formed from cholesterol in HDL and transferred to IDL (Ganong, 2001; Fielding and Fielding, 2002). Consequently, the LDL produced from IDL contains a relatively high amount of cholesteryl esters; approximately 65-70% of plasma cholesterol circulates in LDL. The single protein component of LDL is the non-exchangeable apoB-100 that binds to the LDL receptor (LDLR) expressed by hepatocytes and peripheral tissues (Schneider, 2002). In addition to apoB-100, LDLR has a high affinity for apoE (Russell et al., 1989). Lipoproteins that contain these apolipoproteins (e.g. LDL and VLDL) are internalized upon binding to LDLR. LDLR-mediated endocytosis of circulating plasma LDL is the predominant process that maintains plasma cholesterol homeostasis (for review see Brown and Goldstein, 1986).

Insect lipoproteins: lipophorins

In contrast to mammals, insects make use of a single type of lipoprotein, which was called lipophorin (Lp) to distinguish the particle from mammalian lipoproteins. Lp effects the transport of lipids through the insect blood (for reviews see Van der Horst, 1990; Ryan and Van der Horst, 2000). The insect blood (hemolymph) generally contains abundant amounts of high-density lipophorin (HDLp) produced by the fat body, a tissue combining many of the functions of mammalian liver on the one hand and adipose tissue on the other (for reviews see Dean and Locke, 1985; Locke, 1998). HDLp has a density similar to that of mammalian HDL (1.09-1.18 g/ml; ∅ 150-170 Å), and carries diacylglycerol (DAG) as its prevalent lipid component in addition to phospholipids, sterols and hydrocarbons (for review see Soulages and Wells, 1994). A characteristic feature of HDLp is its ability to function as a reusable shuttle for a variety of lipids by selective loading and unloading of lipid components at target tissues (for reviews see Van der Horst, 1990; Ryan, 1990; Van der Horst et al., 1993; Ryan and Van der Horst, 2000). In the insect at rest, organs and tissues extracellularly extract lipids such as DAG-derived FFAs from circulating HDLp, the lipid cargo of which is restored by DAG mobilized from fat body TAG reserves (Fig. 2).

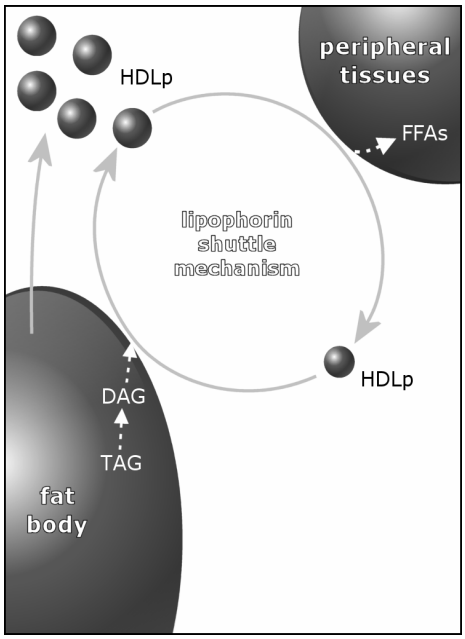

Figure 2. Schematic overview of the lipophorin shuttle mechanism in *L. migratoria* at rest. Following biosynthesis in the fat body, circulating HDLp delivers lipids to peripheral tissues, after which the delipidated particle takes up lipids from the fat body. Both lipid unloading and reloading occurs without internalization of HDLp.

Flight activity of insects has been shown to provide a fascinating, yet relatively simple model system for studying the regulation of processes involved in energy metabolism during exercise. Active insect flight muscles are among the most energy-demanding tissues known, which is reflected in the extremely high metabolic rates sustained during long-distance flight. Despite many similarities with the mammalian system, including the storage of lipid reserves as TAG and their utilization for the energy-generating process in the muscles as FFAs, particularly the exercise-induced processes of lipid mobilization and transport of lipids appear to be different. In contrast to the situation in insects, hydrolysis of TAG from mammalian adipose tissue results in the release of FFAs, which are transported in the blood bound to albumin, an abundant serum protein harboring several fatty acid binding sites (Spector, 1995). Lipid mobilization in long-distance flying insects such as the migratory locust, *Locusta migratoria* (Fig. 3) has revealed a novel concept for lipid transport involving the multifunctional HDLp (for reviews see Van der Horst, 1990; Van der Horst et al., 2001). Flight activity induces lipid mobilization in the fat body cells, resulting in the release of DAG from TAG stores (Fig. 4). The mobilized lipid is loaded onto preexisting HDLp particles circulating in the hemolymph. Concomitant with this loading process, multiple copies of an exchangeable apolipoprotein, apolipophorin-III (apoLp-III) reversibly associate with the expanding particle surface and finally, the particle is converted into lipid-enriched low-density lipophorin (LDLp). LDLp has a density similar to that of human LDL (1.02-1.07 g/ml; ∅ 250-320 Å) and a high capacity to transport DAG molecules to the flight muscles. Hydrolysis of LDLp-carried DAG at the flight muscles results in dissociation of apoLp-III from the particle until, eventually, HDLp is recovered. Both HDLp and apoLp-III are reutilized for another cycle of lipid uptake and transport, thus acting as an efficient lipid shuttle mechanism. This lipid loading and unloading of lipophorin particles during sustained flight activity requires their internalization by neither donor nor recipient cells and consequently, the

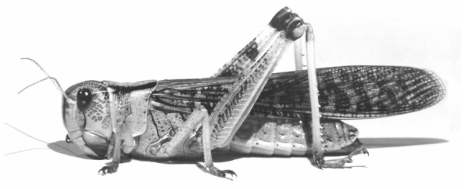

Figure 3. The African migratory locust, *Locusta migratoria*.

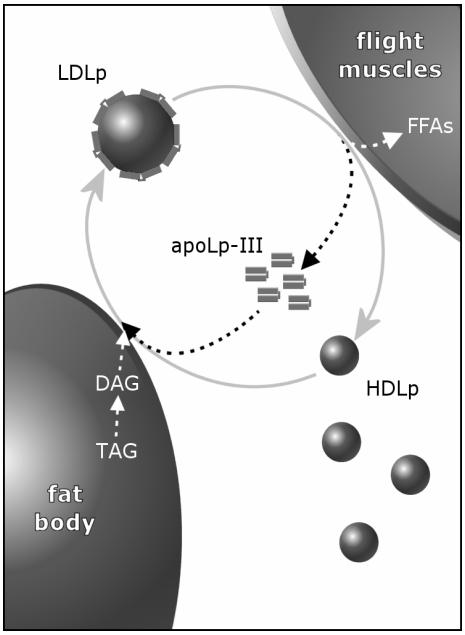

Figure 4. Schematic overview of the transfer of lipids from fat body to flight muscles during sustained flight activity. Circulating HDLp is loaded with additional lipids (DAG) derived from the fat body. During this process, several copies of exchangeable apoLp-III associate with the particle. The resulting lipid-protein complex, LDLp, delivers its lipid cargo to the flight muscles. LDLp-carried DAG is extracellularly hydrolyzed, which triggers the release of the exchangeable apoLp-III molecules. Latter processes occur without internalization of the lipoprotein particles.

increased capacity for lipid transport is achieved without additional lipophorin biosynthesis (for reviews see Van der Horst, 1990; Van der Horst et al., 1993, 2001).

Oviparous animals (e.g. insects and birds) supply their eggs with proteins, carbohydrates and lipids; the latter are transported in combination with proteins predestined for yolk formation. With the exception of a few insect species like higher Diptera (e.g. *Drosophila melanogaster*), the most abundant yolk protein in oviparous species is vitellogenin (Vg). Insect Vgs are synthesized in the fat body as oligomeric lipid-protein complexes, the monomers of which are composed of two subunits derived from the cleavage of a single precursor. In contrast, vertebrate Vgs are synthesized in the liver and are cleaved into several subunits only after internalization by the oocyte (for review see Sappington and Raikhel, 1998).

HDLp *versus* LDL

HDLp generally contains two non-exchangeable apolipoproteins, apolipophorin-I (apoLp-I; ~240 kDa) and apolipophorin-II (apoLp-II; ~80 kDa) that are derived from a common precursor protein through post-translational cleavage (Weers et al., 1993). Molecular characterization of the apolipophorin precursor has recently been disclosed for a few insect species, showing that the protein is arranged with apoLp-II at the N-terminal end and apoLp-I at the C-terminal end (hence also termed apoLp-II/I). Similar to the other apolipophorin precursors known to date, in *L. migratoria* apoLp-II/I (3359 amino acid residues), the N-terminus of apoLp-I is preceded by the amino acid sequence RQKR, suggesting that in the cleavage of the precursor protein, dibasic-processing endoproteases of the subtilisin family are likely involved (Bogerd et al., 2000).

The N-terminal ~900 amino acid residues of apoLp-II/I show significant homology with the same part of human apoB-100 (Fig. 5), its non-exchangeable counterpart in human (V)LDL, invertebrate and vertebrate Vgs, and the large subunit of mammalian microsomal triglyceride transfer protein (Babin et al., 1999; Mann et al., 1999). These homologies indicate that the apolipophorin precursors are members of a large lipid transfer protein superfamily that emerged from an ancestral molecule designed to ensure a pivotal event in the intracellular and extracellular transfer of lipids and liposoluble substances (Babin et al., 1999). In addition, the amino acid residues 2800-3050 show

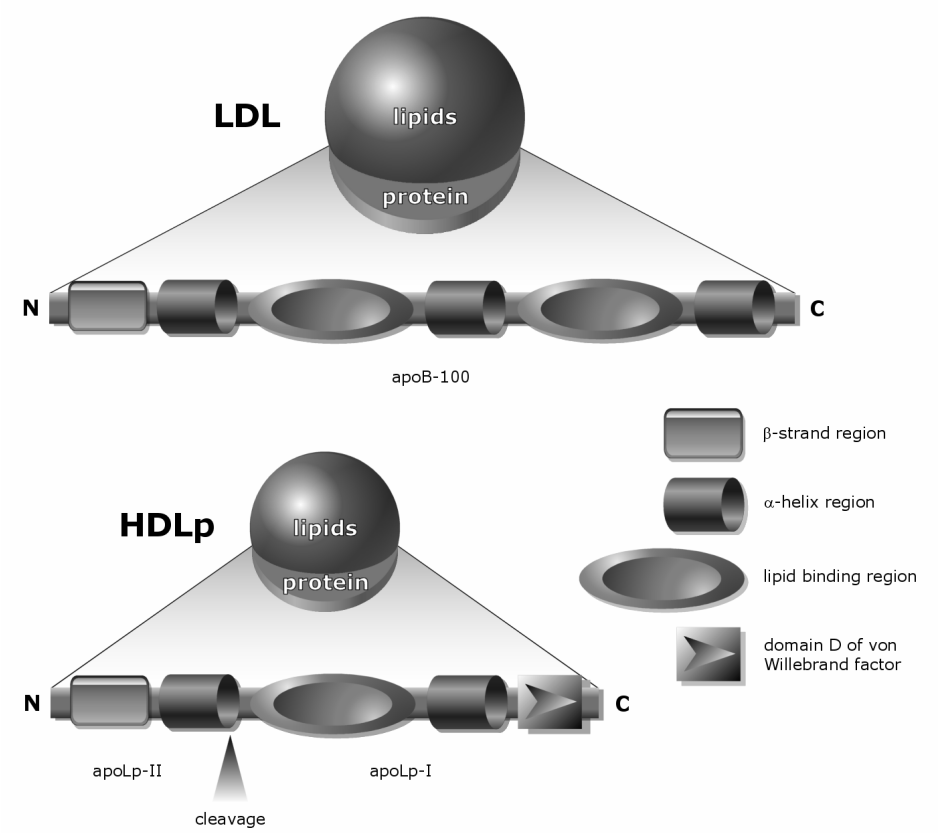

Figure 5. Schematic overview of the protein matrix of LDL and HDLp. LDL contains a single copy of non-exchangeable apoB-100. The non-exchangeable protein component of HDLp consists of a single copy of apoLp-I and apoLp-II, both of which are derived from a common precursor protein through post-translational cleavage.

homology to domain D of the von Willebrand factor (Babin et al., 1999; Bogerd et al., 2000); the function of this domain is as yet unknown. Sequence analysis of other insect apoLp-II/I sequences revealed the presence of a large β-strand-containing domain between residues ~900 and ~2200, which potentially relates to the large lipid-binding domain in apoB-100 termed β-strand motif (Babin et al., 1999; K.W. Rodenburg, unpublished data), two of which are found in the apoB-100 protein (Segrest et al., 2001). Between residues ~2250 and ~2750 in apoLp-II/I, an α-helical domain shows sequence homology to the amphipathic α-helical cluster α2 in apoB-100 (Segrest et al., 2001). ApoLp-II/I is approximately 1200 amino acid residues shorter than apoB-100, the most C-terminal 1000 amino acid residues of which are involved in the binding of LDL to LDLR. Even though the overall domain structure of apoLp-II/I resembles that of apoB-100, no putative receptor-binding site showing homology to this C-terminal part of apoB-100 has been identified in apoLp-II/I.

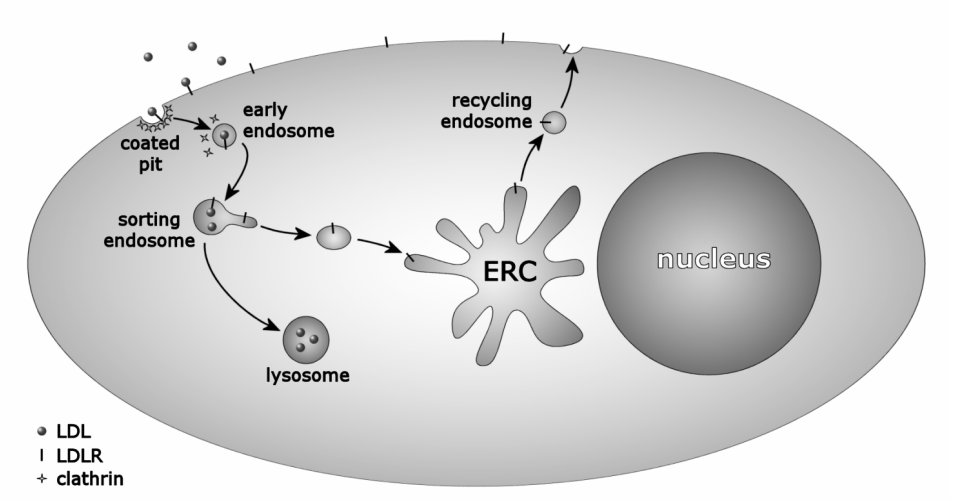

Figure 6. Schematic overview of LDLR-mediated uptake of LDL in mammalian cells. LDL binds to LDLR located in coated pits at the cell surface, after which the receptor-ligand complex is internalized. The clathrin coat is released from the internalized vesicle, which fuses with a preexisting sorting endosome. Therein, LDL is dissociated from LDLR upon acidification of the vesicle lumen. LDL-free LDLR enters the tubules of the sorting endosome. The tubules pinch off and become vesicles that fuse with the ERC, from which membrane-anchored molecules are recycled back to the cell surface. LDL remains in the lumen of the sorting endosome, that matures into a lysosome.

Receptor-mediated endocytosis of LDL

LDL is taken up by cells via receptor-mediated endocytosis involving LDLR (for reviews see Goldstein et al., 1985; Brown and Goldstein, 1986). This receptor is a transmembrane protein with an extracellular N-terminus harboring a ligand-binding domain that can bind lipoproteins containing apoB-100 (e.g. LDL) or apo-E (e.g. VLDL; for review see Mahley and Innerarity, 1983), and an intracellular C-terminus with an FDNPVY internalization signal (Davis et al., 1986a, 1987a; Bansal and Gierasch, 1991; Kibbey et al., 1998). LDLRs present on the cell surface in clathrin-coated pits bind circulating LDL, after which the pits invaginate and pinch off from the cell surface to form clathrin-coated vesicles (Fig. 6; for review see Goldstein et al., 1979). The clathrin coat is released and the vesicle (i.e. early endosome) fuses with other early and recently formed endosomes to become a sorting endosome (for review see Mellman, 1996; Mukherjee et al., 1997). After repeated fusions, the tubulo-vesicular sorting endosome becomes inaccessible to newly internalized vesicles and the lumen is acidified to pH 6.0-6.5 which triggers the release of LDL from LDLR (for reviews see Tycko et al., 1983; Innerarity, 2002; Jeon and Blacklow, 2003). Whereas membrane-spanning LDLRs enter the tubules that bud off to form transport vesicles, the remaining LDL particles are retained in the sorting endosomes. Sorting endosomes mature into lysosomes (Stoorvogel et al., 1991) where enzymes break down the apoB-100, and cleave the ester bonds of cholesteryl esters to yield unesterified cholesterol for membrane synthesis and other cellular needs (for review see Brown and Goldstein, 1986). LDLR-containing transport vesicles derived from the tubular extensions of sorting endosomes fuse with the large, long-lived juxtanuclear endocytic recycling

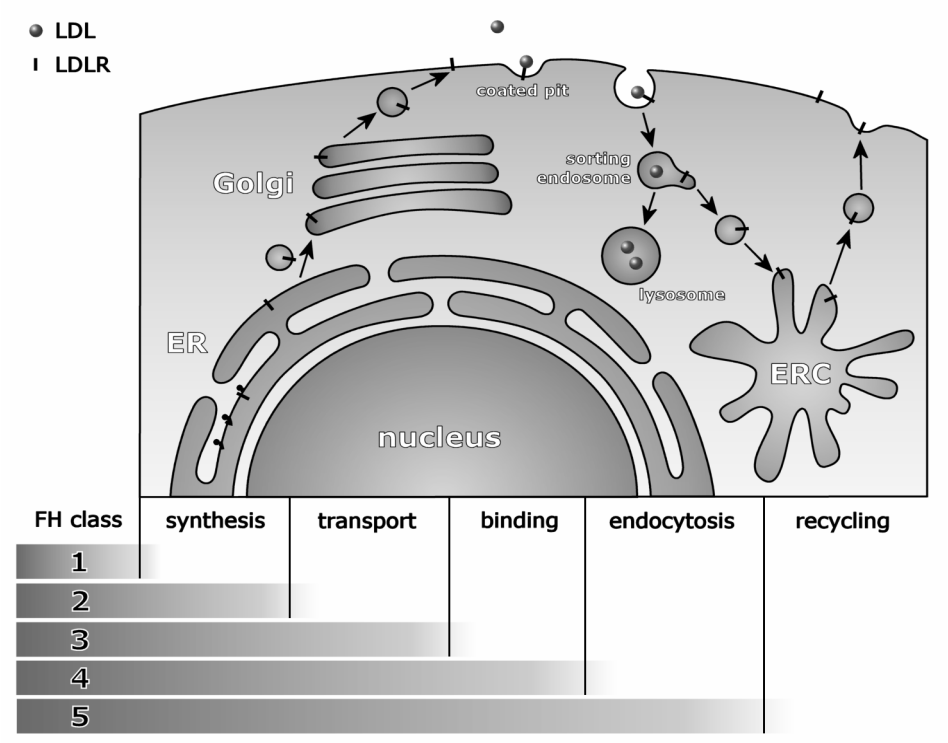

Figure 7. Schematic overview of the different FH mutation classes that impair LDLR expression and functioning. Class 1 mutations impede LDLR synthesis, class 2 mutations prevent or retard transport of LDLR to the cell surface (ER, endoplasmic reticulum), class 3 mutations inhibit binding of LDL to the ligand binding domain of LDLR, class 4 mutations block endocytosis of LDLR, and class 5 mutations impair recycling of LDLR to the cell surface after internalization.

compartment (ERC; Yamashiro et al., 1984) from which many membrane constituents are recycled back to the cell membrane (for review see Maxfield and McGraw, 2004). LDLR thereby escapes the lysosomal fate of LDL and can be used for the binding of another LDL particle.

Disorders in LDL uptake

The prevalent fate of circulating LDL is LDLR-mediated endocytosis by the liver. Humans homozygous for wild-type LDLR have plasma LDL-cholesterol levels of <2 g/l. Reductions in hepatic LDLR number or activity result in elevated plasma levels of LDL-cholesterol. Familial hypercholesterolemia (FH) is an autosomal dominant disorder caused by mutations in the LDLR gene that affect the proper expression or functioning of LDLR *in vivo* (Goldstein and Brown, 1989). In the European population, the incidence of FH is approximately one in five hundred for the heterozygous and one in a million for the homozygous form resulting in plasma LDL-cholesterol levels of ~4 g/l and >10 g/l, respectively (for reviews see Hobbs et al., 1990; 1992). In addition to elevated plasma cholesterol, FH patients are clinically characterized by increased incidence of premature ischemic heart disease and tendon xanthomas (Tabas, 2002). At

present, more than 700 mutations have been identified in the LDLR locus (http://www.ucl.ac.uk/fh), and are subdivided into five different classes that lead to: (1) no immunoprecipitable LDLR protein, (2) no or delayed transport of (misfolded) receptor protein from endoplasmic reticulum to Golgi, (3) expression of cell surface receptors that are incapable of binding LDL, (4) LDL-binding receptors that fail to enter the cell, and (5) LDL-internalizing receptors that do not return to the cell surface after endocytosis. A schematic overview of the subdivision of these mutations is presented in Fig. 7.

LDLR gene and protein structure

LDLR is the representative prototype for a large class of endocytic transmembrane receptors (for reviews see Willnow, 1999; Hussain et al., 1999; Hussain, 2001). Since its discovery in 1973 by Goldstein and Brown, for which they were awarded the Nobel Prize in 1985, many LDLR homologues have been identified and characterized in man and other species (Table 1). LDLR was first purified from bovine adrenal cortex (Schneider et al., 1982), the amino acid sequence (Russell et al., 1983) of which was used to obtain the full-length cDNA for human LDLR (Yamamoto et al., 1984). The 839 amino acid residues long mature receptor is composed of five characteristic domains (Fig. 8): (1) an N-terminally-located ligand binding domain, (2) an epidermal

Table 1. Vertebrate LDLR family members.

Receptor	Species	Accession #
LDLR	*Homo sapiens* (human)	NP_000518
	Mus musculus (house mouse)	QRMSLD
	Rattus norvegicus (Norway rat)	NP_786938
	Cricetulus griseus (Chinese hamster)	P35950
	Oryctolagus cuniculus (rabbit)	P20063
	Sus scrofa (pig)	AAC39254
	Xenopus laevis (African clawed frog)	Q99087
	Danio rerio (zebrafish)	AAP22970
	Chiloscyllium plagiosum (whitespotted bambooshark)	AAB42184
VLDLR	*Homo sapiens* (human)	NP_003374
	Mus musculus (house mouse)	P98156
	Rattus norvegicus (Norway rat)	NP_037287
	Oryctolagus cuniculus (rabbit)	P35953
	Bos taurus (cow)	NP_776914
	Xenopus laevis (African clawed frog)	JC4858
LR8B	*Gallus gallus* (chicken)	CAA65729
LRP	*Homo sapiens* (human)	NP_002323
	Mus musculus (House mouse)	NP_032538
	Gallus gallus (chicken)	P98157
	Caenorhabditis elegans (nematode)	NP_492127
LRP-2	*Homo sapiens* (human)	NP_004516
	Mus musculus (house mouse)	XP_130308
	Rattus norvegicus (Norway rat)	NP_110454
LRP-8	*Homo sapiens* (human)	NP_004622
	Mus musculus (house mouse)	NP_444303
VgR	*Gallus gallus* (chicken)	P98165
	Oreochromis aureus (blue tilapia)	AAO27569
	Oncorhynchus mykiss (rainbow trout)	CAD10640
SLR	*Oncorhynchus mykiss* (rainbow trout)	CAA05874

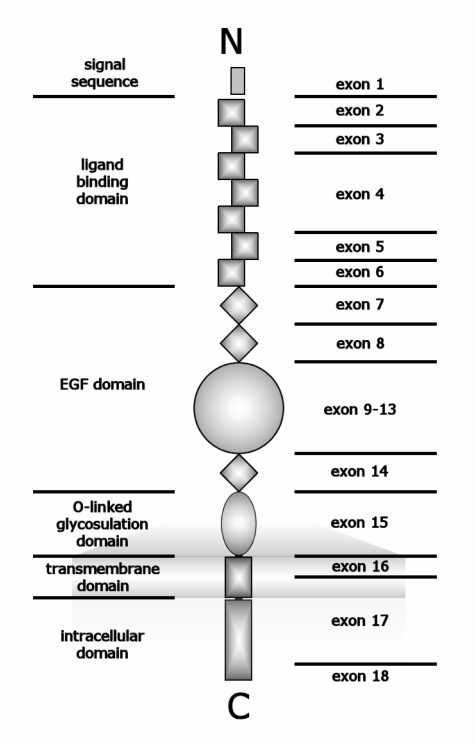

Figure 8. Schematic model of wild-type LDLR. The receptor is composed of a ligand binding domain harboring 7 CRs (squares), an EGF domain comprising two consecutive EGF repeats (diamonds) that are separated from a third by a β-propeller (circle), an O-linked glycosylation domain (oval), a transmembrane domain (short rectangle), and an intracellular cytoplasmic tail (long rectangle). The exons encoding the regions indicated on the left are depicted on the right.

growth factor (EGF) precursor homology domain, (3) a O-linked glycosylation domain, (4) a single membrane-spanning transmembrane domain, and (5) a C-terminal cytoplasmic tail.

The LDL receptor gene is located on chromosome 19 (Francke et al., 1984) and spans approximately 45 kilobases with 18 exons separated by 17 introns (Sudhof et al., 1985a). The first exon encodes the signal sequence (Fig. 8) preceding the ligand binding domain (292 amino acids) which is encoded by exons 2 to 6, and consists of seven consecutive imperfect cysteine-rich repeats (CRs) of approximately 40 amino acids each. The individual repeats harbor six cysteine residues, the first residue of which is connected to the fourth, the second to the sixth, and third to the fifth via disulfide bonds (Daly et al., 1995a, b). CR 3 to 7 are most likely responsible for binding LDL (Esser et al., 1988), whereas only CR 5 appears crucial for apoE binding (Russell et al., 1989). All CRs bind a single Ca^{2+} ion that appears to be essential for ligand binding (Schneider et al., 1979; Fass et al., 1997; Simmons et al., 1997). In addition to EDTA, which removes the Ca^{2+} ions, also suramin has been shown to inhibit ligand binding (Schneider et al., 1982; George et al., 1987). The EGF domain (417 amino acids), encoded by exon 7 to 14, is 35% homologous to a portion of the extracellular domain of the precursor for EGF (Yamamoto et al., 1984; Sudhof et al., 1985b). It comprises a pair of EGF-like modules (EGF-A and EGF-B; Malby et al., 2001; Kurniawan et al., 2001; Saha et al., 2001) of approximately 40 amino acid residues, separated from a third (EGF-C) module by a series of six YWTD motifs. Similar to the ligand binding repeats, the EGF-like modules contain six cysteine residues each; however, their disulfide bond arrangement differs from the ligand binding repeats in that the first is connected to the third, the second to the fourth, and the fifth to the sixth (Stenflo et al., 2000). The individual YWTD motifs are part of six β-sheets arranged in a β-propeller configuration (Springer, 1998; Jeon et al., 2001) that is proposed to control the pH-dependent ligand dissociation (Davis et al., 1987b; Rudenko et al., 2002). The glycosylation domain (58 amino acids), encoded by a single exon (exon 15), lies immediately external to the cell membrane and contains clustered O-linked sugar chains (Russell et al., 1984). The receptor appears to be heavily

glycosylated, increasing the protein molecular weight of approximately 100 kDa to 160 kDa. The function of this prominent domain is unclear except for suggestions to serve as a spacer, and to render stability. Deletion of this domain does not impair the function of LDLR as observed in transfected fibroblasts (Davis et al., 1986b). The transmembrane domain (22 amino acids) is encoded by exon 16 and the first part of exon 17, and consists of a stretch of hydrophobic residues arranged most likely in a helix bundle. The intracellular tail (50 amino acids), encoded by the second part of exon 17 and exon 18, plays an essential role in clustering LDLR into coated pits via the FDNPVY motif. This motif can interact with clathrin in a reverse-turn conformation (Bansal and Gierasch, 1991; Kibbey et al., 1998), and is most likely also involved in cellular signaling (for reviews see Herz et al., 2000; Bonifacino and Traub, 2003).

Mechanism of ligand binding and uncoupling; FH class 5 LDLR mutations

The functions of several individual receptor domains are evident from naturally occurring mutations in the LDLR gene that result in FH, and have been described and characterized in detail (for review see Hobbs et al., 1992; http://www.ucl.ac.uk/fh). Generally, mutations in the promoter and N-terminal region of the receptor that result in early stop-codons, e.g. in the case of point mutations, deletions or insertions, are defined as null alleles. Normally, protein processing takes place in the Golgi within 60 min after synthesis. Transport-defective alleles that give rise to misfolded proteins which are blocked from transit to the Golgi cannot be assigned to a specific domain, but predominantly occur in the EGF precursor region, and constitute approximately half of the identified FH mutations (for review see Hobbs et al., 1992). Recombinant deletion of the first CR has no effect on LDL binding (Van Driel et al., 1987); however, removal of any other repeat as well as substitution of single conserved amino acid in the ligand binding domain results in 95% loss of binding. Internalization mutations are rare but typically involve the clathrin-binding sequence FDNPVY (Davis et al., 1986a, 1987a). Whether this motif directly interacts with clathrin (Kibbey et al., 1998) or indirectly via adaptor proteins (e.g. autosomal recessive hypercholesterolemia; Garcia et al., 2001) is still unclear, but it is undoubtedly essential for endocytosis of the receptor. The most recently characterized class of FH mutations involves impaired receptor recycling. It is assumed that if LDLR fails to release its ligand in the endosome, the complex is degraded resulting in a decrease of cell surface LDLRs (Beisiegel et al., 1981a, b; Davis et al., 1987b; Miyake et al., 1989), and eventually leads to hypercholesterolemia.

In 1989, the first naturally occurring FH mutant with impaired recycling ability was reported (Miyake et al., 1989). Two years earlier, Davis et al. (1987b) had constructed an LDLR mutant, the complete EGF precursor homology domain of which was deleted (LDLRΔEGF; removal of amino acids G293 to T692). This receptor did not bind LDL, whereas VLDL binding was maintained. LDLRΔEGF showed complete loss of its ability to dissociate ligand (i.e. VLDL) at acid pH, with consequent impairment of recycling of the receptor to the cell surface. From these results it was concluded that the EGF domain is essential for recycling of the receptor to the cell surface. This hypothesis was supported by the characterization of a homozygous deletion mutant, exons 7-14 of which were absent from the gene (Miyake et al., 1989). This naturally occurring mutant is identical to the recombinant LDLRΔEGF mutant produced *in vitro* (Davis et al., 1987b), and results in FH *in vivo* (Miyake et al., 1989). More precisely, deletion of EGF-A and EGF-B is sufficient to impair LDL binding and receptor recycling, as

shown for a naturally occurring FH mutation in which exon 7 and 8 are deleted (Van der Westhuyzen et al., 1991).

Attempts have been made to elucidate the molecular mechanism behind impaired receptor recycling and the relation with the inability of the receptor to dissociate bound ligand in the sorting endosome. Recently, the X-ray crystal structure at 3.7 Å resolution of a part of the LDLR extracellular domain was solved at pH 5.3, which was proposed to represent the conformation of LDLR adopted in endosomes (Rudenko et al., 2002). The extracellular domain comprised amino acid residues 1 to 699, representing CR 1 to 7 of the ligand binding domain, EGF-A and EGF-B, followed by the β-propeller and EGF-C (Fig. 9A). In the crystal structure, the ligand binding domain forms an arc that folds back onto the EGF domain where CR 4 (Cys127 to Cys163) and CR 5 (Cys176 to Cys210) bind to the β-propeller (Ile377 to Gly642) via extensive interactions (Fig. 9B). These interactions include hydrophobic contacts and salt bridges between CR 4 and the β-propeller, as well as between CR 5 and the β-propeller (Fig. 9C).

Histidine residues, located in CR 5 (His190) and the β-propeller (His562 and His586), are ideally suited to act as a pH-sensitive switch. The pK_a of His in proteins is 6.7, and therefore harbor a positive charge at slightly acidic pH (e.g. in endosomes). The conformation of the LDLR ectodomain at low pH, in which the key contributors to LDL binding (CR 4 and 5) are engaged in intramolecular contacts with the β-propeller, suggests that the β-propeller serves as an alternate ligand in the sorting endosome (for reviews see Innerarity, 2002; Jeon and Blacklow, 2003). Naturally occurring FH mutations in two of the three histidines at the interface, H190Y (Hopkins et al., 1999) and H562Y (Sun et al., 1994; J.C. Defesche, 2003, personal communication), support the involvement of these histidine residues in pH-induced ligand uncoupling and suggest that these receptor mutants are unable to dissociate LDL in the endosome.

Whether ligand release and conformational change are two independent steps, or ligand dissociation is induced by the conformational change remains to be elucidated. In any case, dissociation of ligand appears essential for cholesterol homeostasis, as mutations that occur in the EGF domain disrupting the ligand uncoupling ability of LDLR, result in elevated plasma LDL-cholesterol. This phenotype can be explained by the recycling property of LDLR. Normally, LDLR is constitutively internalized and recycled with or without antecedent ligand binding (Brown et al., 1982). Each wild-type LDLR recycles approximately 150 times before being degraded in lysosomes. During its ~30-hour life span (for review see Goldstein et al., 1979), each receptor is capable of internalizing many LDL particles, thus reducing the number of receptors that need to be produced by the cell. However, when the receptor is unable to release its ligand (i.e. LDL) after internalization, the complex is transported to lysosomes (Davis et al., 1987b), as also demonstrated with a polyclonal anti-receptor IgG (Beisiegel et al., 1981a, b); both ligand and receptor are degraded, resulting in a decrease in the number of receptors. As a consequence, the amount of plasma LDL-cholesterol remains elevated.

Vertebrate LDLR homologues

The LDLR domains discussed above are present in all members that belong to the LDLR family (for review see Hussain, 2001), which can be divided into two subgroups according to structural organization of their extracellular receptor domains: (1) low molecular weight (LMW) receptors with a single ligand binding and EGF domain, and

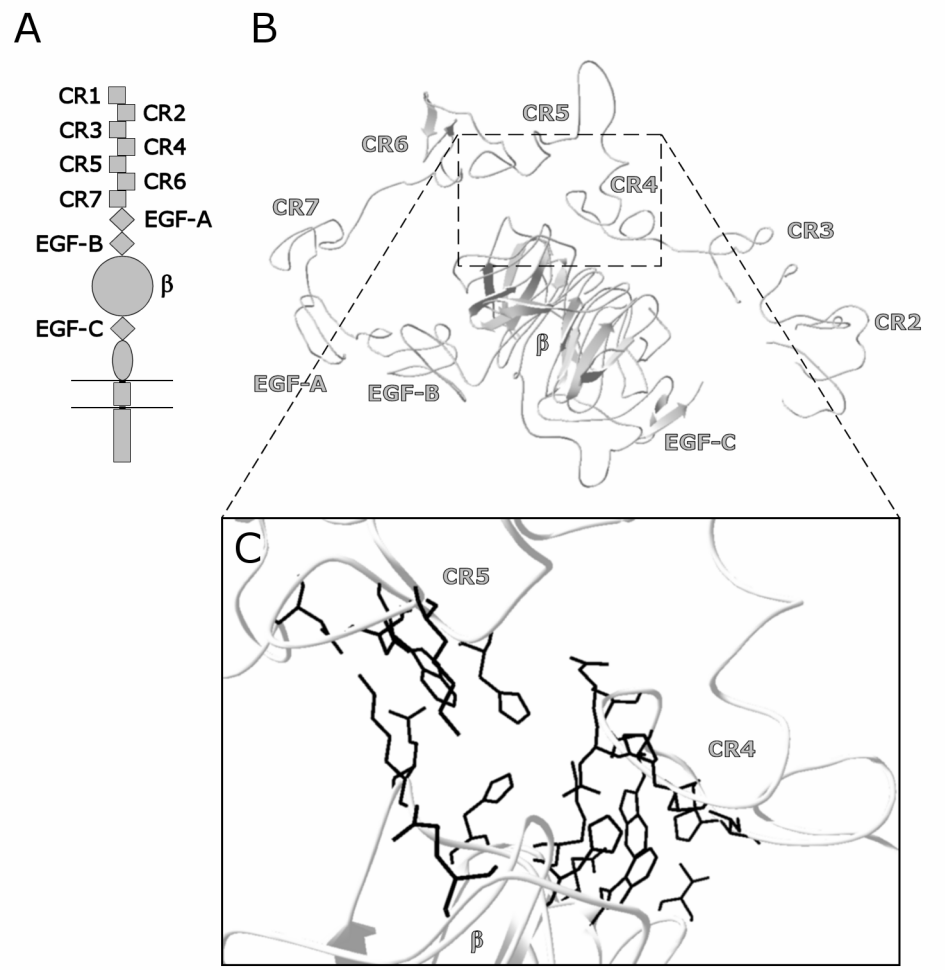

Figure 9. Schematic model of the conformation of wild-type LDLR at neutral pH (A). Three-dimensional ribbon model of the X-ray crystal structure at 3.7 Å resolution of a part of the LDLR extracellular domain at pH 5.3 (B). CR 1 is present but not seen because of disorder in the crystal. (C) Enlargement of the box in (A) showing the amino acid residues (back bone and side chain of each residue is indicated in black) that interact in the interface between CR 4 and 5 of the ligand binding domain and the β-propeller of the EGF domain. Swiss PDB Viewer 3.7 (http://swissmodel. expasy.org/) (Guex and Peitsch, 1997; Schwede et al., 2003) was used to generate the three-dimensional reconstruction of the LDLR ectodomain (PDB entry 1N7D).

(2) high molecular weight (HMW) receptors with multiple copies of these two extracellular domains.

VLDLR belongs to the LMW LDLR family members. This receptor has a domain arrangement similar to LDLR, and is synthesized as a 135 kDa precursor that is converted to a glycosylated 155 kDa mature receptor. VLDLR was originally isolated from a rabbit heart cDNA library (Takahashi et al., 1992), and cloned on the basis of its homology to LDLR. In contrast to LDLR, VLDLR has one additional CR at the N-terminus of the ligand binding domain, and is expressed in extrahepatic tissues (e.g.

heart, muscle and adipose tissue). Like LDLR, it shows affinity for apoE-containing lipoproteins (e.g. VLDL, IDL and chylomicrons); however, it does not bind LDL. In addition to VLDL, this receptor showed high affinity for receptor-associated protein (RAP; Battey et al., 1994; Simonsen et al., 1994), a potent competitor for lipoproteins. RAP is a 39 kDa protein that is assumed to function intracellularly as a chaperone to assist the folding of several LDLR family members (Bu et al., 1995; Bu and Rennke, 1996; Bu and Schwartz, 1998; Bu and Marzolo, 2000) which do not include LDLR, as RAP has only weak affinity for this receptor (Medh et al., 1995). Although the biological significance is still poorly understood, binding to VLDLR was also shown for LPL (Takahashi et al., 1995), thrombospondin-1 (Mikhailenko et al., 1997), urokinase plasminogen activator-plasminogen activator inhibitor-1 (PA:PAI-1) complex (Argraves et al., 1995; Heegaard et al., 1995), and other proteinase-serpin complexes (Kasza et al., 1997). The role of VLDLR in lipoprotein metabolism initially proved difficult to unravel because studies in VLDLR-knock out mice revealed no alterations in plasma lipid and lipoprotein levels, and only a small reduction in adipose tissue mass (Frykman et al., 1995). However, absence of both LDLR and VLDLR in double knock out mice was associated with increased VLDL-TAG levels, whereas endothelial VLDLR overexpression in these double knock out mice was associated with decreased VLDL-TAG levels, suggesting that VLDLR does indeed play a role in VLDL-TAG metabolism (Tacken et al., 2000).

A somatic specific 130 kDa homologue of VLDLR, harboring a ligand binding domain with eight CRs has been identified in chicken (LR8; Hayashi et al., 1989). Recently, two other VLDLR homologues have been characterized in chicken as well as mouse (LR8B; Novak et al., 1996). In contrast to all previously identified VLDLRs, the receptor transcripts of these LR8Bs are present at high levels in brain and at much lower levels in extraoocytic cells of the ovary, but absent in heart.

A remarkable feature of VLDLR is that, despite the presence of the FDNPVY motif in the cytoplasmic domain, it is assumed to function extracellularly by mediating selective fatty acid entry into tissues. However, overexpression of VLDLR in transfected Chinese hamster ovary (CHO) cells (Takahashi et al., 1992), as well as ectopic *in vivo* expression of this receptor in mouse liver showed internalization of lipoproteins (Kobayashi et al., 1996; Van Dijk et al., 1998). VLDLR apparently has the ability to function as an endocytic receptor, like all LDLR family members, but principally facilitates extracellular hydrolysis of TAG when endogenously expressed in adipose tissue.

Additional support for the role of the EGF domain in ligand dissociation was obtained from studies with VLDLR. CHO cells that were transfected with VLDLR were capable of VLDL and RAP endocytosis (Takahashi et al., 1992 and Mikhailenko et al., 1999, respectively). Similar to the lysosomal fate of ligands endocytosed by LDLR, VLDLR-internalized RAP is transported to lysosomes after dissociation from VLDLR in sorting endosomes. However, when the EGF domain of VLDLR is removed, RAP is no longer dissociated and recycles back to the cell surface (Mikhailenko et al., 1999).

LDLR-related protein (LRP, also denoted as α_2-macroglobulin receptor) was the first receptor identified as an LDLR homologue and classified as a member of the family (Herz et al., 1988). With 600 kDa, it is a HMW receptor that is expressed in various mammalian cells, but most abundantly in hepatocytes, fibroblasts and neurons (Herz et al., 1988; Moestrup et al., 1992). LRP is synthesized as a single polypeptide of 4525 amino acids and is cleaved by furin in the Golgi complex to produce two non-

covalently attached subunits of 515 kDa and 85 kDa (Herz et al., 1990; Willnow et al., 1996a). In contrast to LMW receptors, LRP has four ligand binding domains separated by three EGF domains, and concluded by a fourth domain containing only EGF-type repeats that are divided over the 515 kDa and 85 kDa subunits after furin cleavage.

Thus far, no ligands have been identified that bind to the first ligand binding domain comprising two CRs. The second, third and fourth ligand binding domain harboring eight, ten and eleven CRs, respectively, bind a diverse array of structurally unrelated ligands (Croy et al., 2003). With the exception of the first ligand binding domain, RAP has been shown to bind the three other ligand binding domains (Williams et al., 1992). RAP proved essential for the proper folding of LRP and prevents premature binding of ligands to the receptor (Willnow et al., 1994, 1996b; Bu et al., 1995; Bu and Rennke, 1996). In addition to RAP, apoE-containing lipoproteins, LPL and hepatic lipase, proteinase inhibitors and complexes, serine proteinases, matrix proteins and Kunitz-type inhibitors have been shown to bind to LRP (for review see Neels et al., 1998). After binding to LRP, the ligands are internalized and dissociated from the receptor in sorting endosomes, and are sorted to lysosomes for degradation. Like all LDLR family members, LRP recycles back to the cell surface where it is once again available to bind ligands. The interaction of LRP with such a multitude of structurally unrelated ligands suggests a role for the receptor in diverse physiological processes, e.g. lipoprotein metabolism, cell growth and cell migration, embryonic development, atherosclerosis and Alzheimer's disease.

Comprising 4660 amino acids, LRP-2 is a single 330 kDa glycoprotein, which was also denoted as glycoprotein 330 (Kerjaschki and Farquhar, 1983) or megalin, and constitutes the largest mammalian member of the LDLR family known to date (Saito et al., 1994). LRP-2 has four ligand binding repeats of seven, eight, ten and eleven CRs, respectively, alternated by four EGF domains. Although *in vitro* it binds many of the ligands that also bind to LRP, its temporal and spatial expression pattern differs significantly from that of LRP. It is primarily expressed in the absorptive epithelial cells of the intestine, proximal tubules of the kidney, lung, and ventricular system of the brain (Zheng et al., 1994). LRP-2-deficient mice die within 2-3 h after birth due to defects in pulmonary inflation and alveolar expansion. In addition, these animals are characterized by abnormal formation of the forebrain and related structures including the eyes, ventricular system, corpus callosum forebrain hemispheres, and lack of olfactory bulbs (Willnow et al., 1996c).

Although the single ligand binding domain of the LMW receptor LRP-8 harbors seven CRs, it is more homologous to VLDLR than LDLR. LRP-8 is mainly expressed in the brain and shows high affinity for apoE-containing lipoproteins, hence also termed apoE receptor-2 (Kim et al., 1996). When expressed in cultured cells, LRP-8 acts as an endocytic receptor for lipoproteins. However, in the embryonic brain it serves as a receptor for reelin, a signaling factor that controls neuronal positioning during development (D'Arcangelo et al., 1999). Binding of reelin to LRP-8 or VLDLR (Trommsdorff et al., 1999; Hiesberger et al., 1999) results in phosphorylation of tyrosine residues in disabled-1, a cytosolic adapter protein that associates with the NPxY motif (with x representing any amino acid) of the receptor cytoplasmic tail (Howell et al., 1997; Trommsdorff et al., 1998). Phosphorylated disabled-1 interacts with SH2 domains of tyrosine kinases of the Abl, Fyn and Src family. This binding plays an important role in the amplification of signals essential for proper development of the brain (Rice et al., 1998). Mutations in reelin or disabled-1 result in disruption of

laminar structure throughout the brain, a phenotype that is also observed in mice lacking functional VLDLR and LRP-8 receptors (Trommsdorf et al., 1999). Although RAP binds to LRP-8, no resulting cellular signaling event has been reported.

Several related vertebrate receptors that share LDLR ligand binding domain similarity have been identified in birds, like two chicken LRPs of 515 kDa and 380 kDa that are expressed in liver and ovarian follicles, respectively (Stifani et al., 1991), and a 95 kDa oocyte-specific splice variant of the 130 kDa somatic chicken VLDLR homologue LR8 (Stifani et al., 1990; Barber et al., 1991; Bujo et al., 1994). This 95 kDa receptor was shown to bind Vg, hence also termed VgR (Barber et al., 1991). Mutations in the VgR gene result in female sterility due to failure in the deposition of egg yolk proteins (Bujo et al., 1994, Bujo et al., 1995). In addition, these birds develop severe hyperlipidemia associated with premature atherosclerosis (Bujo et al., 1995). In addition to birds, LDLR family members have also been identified in fish (Prat et al., 1998; Li et al., 2003). Apparently, CRs are universally occurring amino acid stretches, although their roles may differ and be specific for each protein. The ligand binding domain is typical for each LDLR family member; however, the intracellular domain is also unique and may be involved in several signalling processes (for review see Herz and Bock, 2002). Receptors that are able to bind RAP, but do not share any sequence homology with LDLR family members have also been reported (e.g. sortilin; Petersen et al., 1997). In addition to RAP, sortilin was observed to bind LPL and neurotensin (Nielsen et al., 1999).

Insect LDLR homologues

LDLR family members have been identified and characterized in many different vertebrates including mammals, amphibeae and fish (Table 1), and invertebrates such as nematodes and insects. Because HMW receptors have been found in *Caenorhabditis elegans* (Yochem and Greenwald 1993; Grant and Hirsh 1999), the large receptors are believed to be phylogenetically older members of the LDLR family. The small receptors that are found in vertebrates and insects may have evolved later in evolution by truncation of larger receptor genes.

Oocyte-specific receptors that belong to the LDLR family and specifically mediate the uptake of yolk constituents are expressed in oviparous vertebrates (Bujo et al., 1994; Okabayashi et al., 1996; Prat et al., 1998; Davail et al., 1998; Li et al., 2003) and insects (for review see Sappington and Raikhel, 1998), and in both classes naturally occurring receptor mutants have been identified that cause female sterility (Bujo et al., 1995; DiMario and Mahowald 1987). Insect VgRs are HMW receptors that have two EGF domains, and two ligand binding domains comprising five and seven CR clusters, respectively. The similarity of *D. melanogaster* yolk protein receptor Yl to the other LDLR proteins is restricted to the extracellular domain; moreover, the cytoplasmic domain of Yl lacks the typical NPxY sequence (Schonbaum et al., 1995). Other VgRs have been identified in several insects (for review see Sappington and Raikhel, 1998), two of which have been sequenced: *Aedes aegypti* (Sappington et al., 1996) and *Periplaneta americana* (Tufail and Takeda, 2002; published only in database) VgR. These receptors appear to function as endocytic receptors for Vg and recycle after endocytosis in the oocyte, as shown for VgR of *A. aegypti* (Snigirevskaya et al., 1997).

In 1990, the first lipophorin-specific receptor was biochemically identified in the fat body of *Manduca sexta* (Tsuchida and Wells 1990). Although it shared some properties characteristic for vertebrate LDLR family members (e.g. molecular weight,

requirement for Ca^{2+} and inhibition of ligand binding by suramin), it was suggested to function extracellularly to assist the transfer of lipids from lipophorin to the fat body (Tsuchida and Wells 1990; Canavoso et al., 2003).

A novel insect member of the LDLR family was identified in *L. migratoria* and sequenced (Dantuma et al., 1999). The involvement of a receptor in the binding of HDLp and uptake of lipids by the fat body had already been proposed earlier (Dantuma et al., 1996, 1997, 1998). The insect LDLR family member cloned from fat body tissue was shown to mediate uptake of lipids from HDLp in transiently-transfected African green monkey kidney (COS-7) cells (Dantuma et al., 1999) and therefore assumed to be involved in the transfer of lipids from HDLp to the insect fat body. This lipophorin receptor (LpR, also denoted as iLR) of 883 amino acids (including a signal peptide of 33 amino acids) with a theoretical molecular weight of ~100 kDa is 39.9% homologous and 69.5% similar to human LDLR, displaying the highest similarity between the two most conserved extracellular domains: the ligand binding and EGF domains (43.3% homology and 73.0% similarity). Like in VLDLR, the ligand binding domain of LpR is composed of eight CRs, and its overall homology and similarity to VLDLR are 42.7% and 74.2%, respectively. Based on three-dimensional structures of the LDLR domains elucidated by crystallography and NMR (Daly et al., 1995a, b; Fass et al., 1997; Bieri et al., 1998; North and Blacklow, 1999, 2000; Clayton et al., 2000; Beglova et al., 2001; Jeon et al., 2001; Kurniawan et al., 2001; Saha et al., 2001), by virtue of the high amino acid sequence similarity, three-dimensional models of respective LpR domains could be generated of all CRs and the individual EGF domains including the β-propeller (Fig. 10). Expression of LpR mRNA in fat body cells was shown to be down-regulated during adult development (Dantuma et al., 1999), which is consistent with the down-regulation of receptor-mediated internalization of HDLp-derived lipids in fat body tissue (Dantuma et al., 1997). These findings additionally suggest that LpR is not

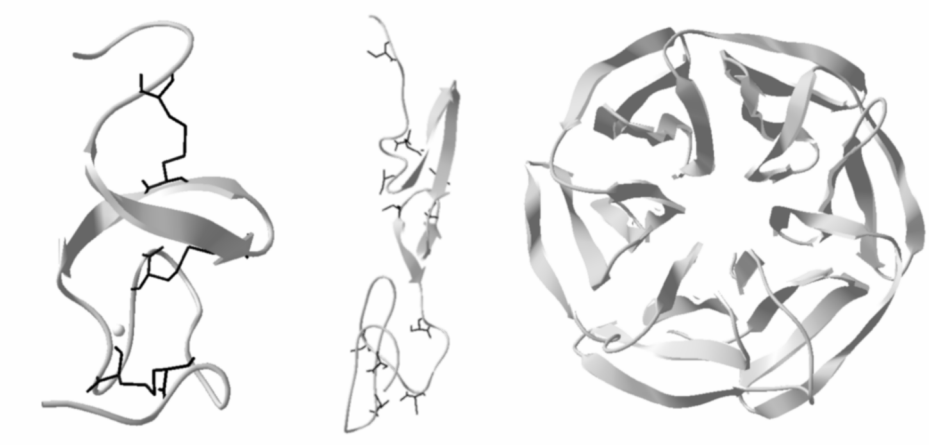

Figure 10. Three-dimensional ribbon model of LpR domains reconstructed with Swiss PDB Viewer 3.7 (http://swissmodel.expasy.org/) (Guex and Peitsch, 1997; Schwede et al., 2003) using elucidated structures of LDLR. First cysteine-rich repeat showing the Cys residues (back bone and side chain of each residue is indicated in black) that form disulfide bonds (left; small sphere, Ca^{2+} ion); EGF-A and EGF-B domain pair showing the Cys residues (back bone and side chain of each residue is indicated in black) that are presumed to form disulfide bonds in the native structure (middle); Six-bladed β-propeller of the EGF domain (right).

involved in the lipophorin shuttle mechanism operative in the flying insect, during which lipids are extracellularly transferred between circulating lipophorin and tissues. In contrast, being a member of the LDLR family suggests that LpR may function as an endocytic receptor for HDLp, although alternative lipid uptake mechanisms operative without endocytosis of the complete lipoprotein have been described for other LDLR family members (i.e. VLDLR), as indicated above. Despite the presence of the FDNPVY internalization motif and its endocytic capacity in transfected COS-7 cells, it remained to be investigated whether LpR can mediate endocytosis of HDLp *in vivo*.

Novel LpRs belonging to the LDLR family that are expressed by the fat body have been identified and characterized in the insects *A. aegypti* (Cheon et al., 2001; Seo et al., 2003) and *Galleria mellonella* (Lee et al., 2003a, b). Putative LDLR homologues were revealed after genome sequencing of *D. melanogaster* (Adams et al., 2000) and *A. gambiae* (Holt et al., 2002; Table 2), and lipophorin receptors expressed by the fat body albeit of unknown sequence and structure have been identified in *M. sexta* (Tsuchida and Wells, 1990) and *Rhodnius prolixus* (Pontes et al., 2002).

Scope of this thesis

Expression of a receptor for HDLp in *L. migratoria* fat body cells is not remarkable in itself, because the particle has to bind to the cell surface for efficient lipid transfer, e.g. lipid reloading by the fat body after delivery of lipids to peripheral tissues in the resting insect, or the formation of LDLp during sustained flight. However, the mRNA isolated from fat body tissue encoded an LDLR family member (i.e. LpR) not only containing a ligand binding domain with putative lipoprotein (i.e. HDLp) binding capacity but, additionally, an internalization signal (i.e. FDNPVY). LDLR family members that are capable of internalizing lipoproteins normally dissociate their ligand in sorting endosomes, after which the ligand is completely degraded in lysosomes. Thus, endocytic uptake of HDLp seems to conflict with the selective process of lipid transfer between HDLp and fat body cells without degradation of the particle. Because LpR mRNA was only present for a few days after the imaginal ecdysis (Dantuma et al., 1999), a period in which the fat body significantly expands in size due to storage of lipids derived from dietary intake, the receptor was expected to be involved in the lipid storage process. The mechanism via which LpR would mediate lipid storage may

Table 2. Invertebrate LDLR family members.

Receptor	Species	Accession #
LRP-1	*Caenorhabditis elegans* (nematode)	NP_492127
RME-2	*Caenorhabditis elegans* (nematode)	NP_500815
Y1	*Drosophila melanogaster* (fruit fly)	P98163
GH26833p	*Drosophila melanogaster* (fruit fly)	AAQ22563
RE38584p	*Drosophila melanogaster* (fruit fly)	AAN71387
VgR	*Aedes aegypti* (yellow fever mosquito) *Periplaneta americana* (cockroach)	T18308 BAC02725
LpR	*Locusta migratoria* (migratory locust) *Aedes aegypti* (yellow fever mosquito) *Anopheles gambiae* (African malaria mosquito) *Galleria mellonella* (wax moth)	CAA03855 AAK72954 XP_308000 n/a*

* The sequence was obtained from Lee et al. (2003a).

include three possibilities: (1) assisting extracellular HDLp-derived lipid hydrolysis to FFAs similar to the proposed role for VLDLR (Fig. 11A), (2) HDLp endocytosis and

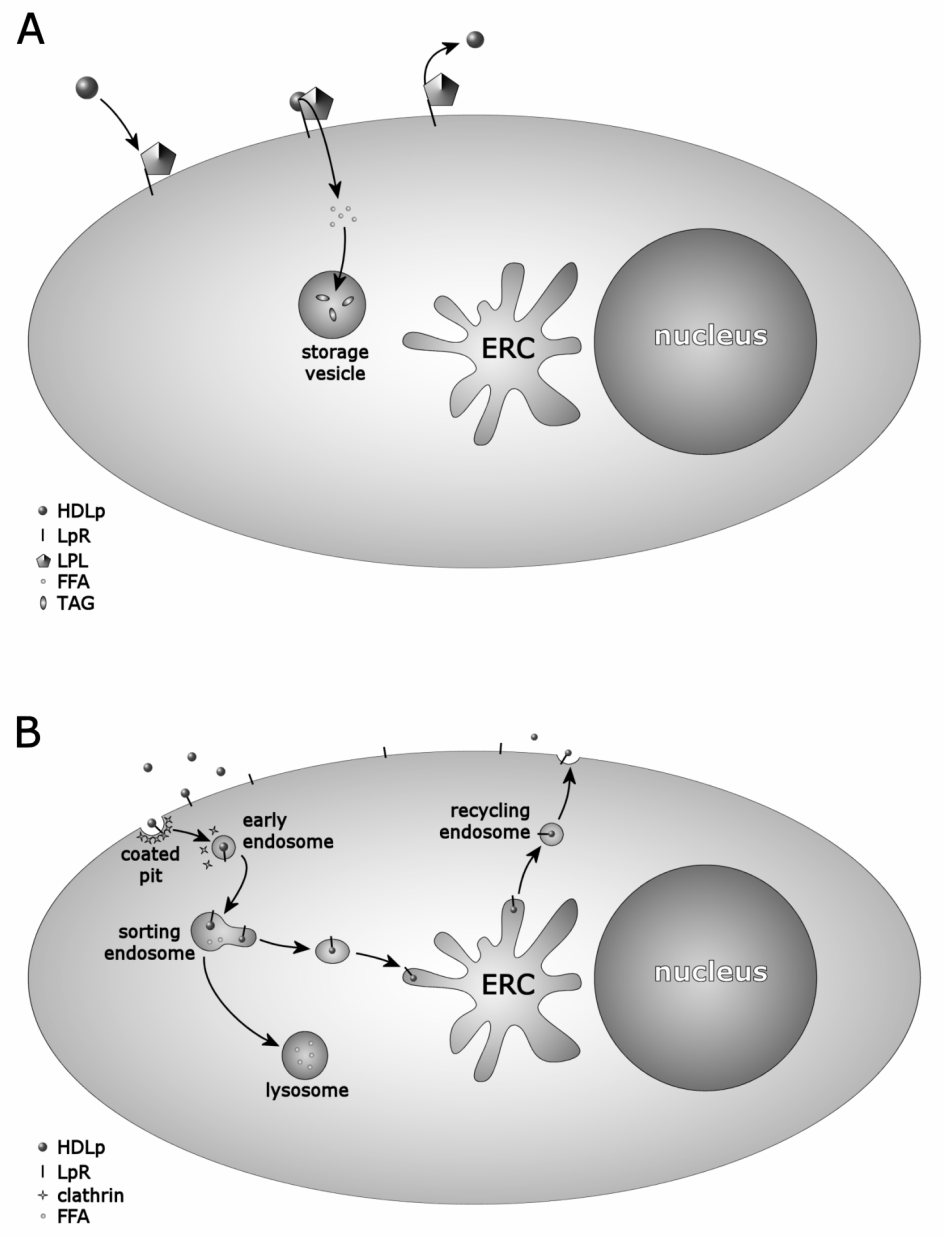

Figure 11. Schematic overviews of hypothetical models for the lipid uptake mechanism by the fat body. (A) Lipids from HDLp are extracellularly converted into FFAs by LPL, after which the released FFAs are taken up by the fat body cells. (B) HDLp is internalized by LpR-mediated endocytosis. Upon internalization, lipids are extracted from HDLp, during which the particle remains attached to LpR. Thereafter, the receptor-ligand complex is recycled in a Tf-like manner.

subsequent degradation of the particle similar to LDLR-mediated LDL uptake (Fig. 6), and (3) endocytosis and subsequent recycling of HDLp after intracellular (partial) delipidation of the particle (Fig. 11B). The latter possibility implies a mechanism that, thus far, has never been described to be mediated by an LDLR family member. On the other hand, latter hypothesis would perfectly comply with the proposal of HDLp functioning as a reusable lipid shuttle.

The ligand specificity, endocytic capacity, and intracellular pathway of LpR as well as its ligands were investigated in a stably-transfected monoclonal CHO cell line using fluorescently-labeled ligands (**Chapter 2**). Intracellular trafficking of LpR-internalized ligands (i.e. Lp and human RAP) was compared to that of LDL and Tf, which follow divergent routes after endocytosis by LDLR and TfR, respectively. The results obtained from these studies revealed that ligands internalized by LpR are not lysosomally degraded like LDL. Instead, LpR-bound ligands follow a Tf-like recycling pathway.

LpR mRNA was isolated from young adult *L. migratoria* fat body tissue. To investigate if LpR mediates endocytosis of ligands in fat body cells, the tissue was incubated *in vitro* with fluorescently-labeled ligands, and analyzed with fluorescence microscopy (**Chapter 3**). These studies showed that Lp and human RAP are only internalized when LpR is expressed. Expression of LpR is down-regulated on the fourth day after an ecdysis, but may be prolonged by starvation of the insects, or even reinduced by applying starvation after down-regulation of LpR expression.

Chapter 4 describes the intracellular pathways of internalized ligands in insect cells. In contrast to the observations in CHO cells, in insect cell lines transfected with LDLR and TfR, LDL and Tf converge in the same vesicular compartments, and remain colocalized during a chase. Similar to Tf, Lp is not recycled from LpR-transfected cells. In contrast, in fat body cells that endogenously express LpR, a chase after incubation with Lp resulted in a significant decrease in Lp-containing vesicles, which is indicative for resecretion of the ligand.

Primary amino acid sequence homology studies based on the LpR region from O-linked glycosylation domain to intracellular tail indicate that LpRs comprise a unique group of LDLR family members (**Chapter 5**). Hybrid receptors composed of LDLR and LpR domains were constructed to identify receptor domains that determine the fate of ligands and receptors after endocytosis. Substitution of the *L. migratoria* LpR intracellular domain by that of LDLR did not alter the fate of Lp or the receptor. On the other hand, after replacing the region of LpR from EGF domain to intracellular tail by that of LDLR, the resulting hybrid receptor recycled neither without, nor in complex with Lp.

To investigate which receptor domains and amino acid residues are responsible for degradation of a dissociation-deficient LDLR, and recycling of dissociation-deficient LpR, the hybrid approach described in Chapter 5 was extended (**Chapter 6**). A naturally occurring FH class 5 LDLR mutant expressed in CHO cells was capable of internalizing LDL. Whereas this mutant receptor was able to recycle in the absence of LDL, its recycling efficiency was significantly reduced upon prolonged LDL incubation. Similar results were obtained with another mutant receptor and a hybrid receptor composed of the ligand binding domain of LDLR and the region from EGF domain to intracellular tail of LpR. The mutant and hybrid studies provide insight into LDLR functioning, and indicate that FH class 5 LDLR mutations can be divided into two subclasses.

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CHAPTER 2

II

Insect lipoprotein follows a transferrin-like recycling pathway that is mediated by the insect LDL receptor homologue

Dennis Van Hoof, Kees W. Rodenburg and Dick J. Van der Horst

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CHAPTER 2

Insect lipoprotein follows a transferrin-like recycling pathway that is mediated by the insect LDL receptor homologue

Dennis Van Hoof, Kees W. Rodenburg and Dick J. Van der Horst

Abstract

The lipoprotein of insects, lipophorin (Lp), is homologous to that of mammalian low-density lipoprotein (LDL) with respect to its apolipoprotein structure. Moreover, an endocytic receptor for Lp has been identified (insect lipophorin receptor, LpR) that is homologous to the LDL receptor. We transfected LDL receptor-expressing CHO cells with LpR cDNA to study the endocytic uptake and intracellular pathways of LDL and Lp simultaneously. Our studies provide evidence that these mammalian and insect lipoproteins follow distinct intracellular routes after receptor-mediated endocytosis. Multicolor imaging and immunofluorescence was used to visualize the intracellular trafficking of fluorescently-labeled ligands in these cells. Upon internalization, mammalian and insect lipoproteins share endocytic vesicles. Subsequently, however, Lp evacuates the LDL-containing endosomes. In contrast to LDL, that is completely degraded in lysosomes after dissociating from its receptor, both Lp and LpR converge in a non-lysosomal juxtanuclear compartment. Colocalization studies with transferrin identified this organelle as the endocytic recycling compartment via which iron-depleted transferrin exits the cell. Fluorescently-labeled human receptor-associated protein (RAP) is also transported to this recycling organelle upon receptor-mediated endocytosis by LpR. Internalized Lp eventually exits the cell via the recycling compartment, a process which can be blocked by monensin, and is resecreted with a $t_{½}$ of ~13 min. From these observations, we conclude that Lp is the first non-exchangeable apolipoprotein-containing lipoprotein that follows a transferrin-like recycling pathway despite the similarities between mammalian and insect lipoproteins and their receptors.

Introduction

The extracellular transport of water-insoluble lipids through the aqueous circulatory system of animals is mediated by lipoproteins. Mammals rely on a wide array of lipoproteins with various compositions and functions. Insects, however, use a single type of lipoprotein, lipophorin (Lp), to effect the transport of a variety of hydrophobic molecules through the blood (hemolymph) (for reviews see Soulages and Wells, 1994; Ryan and Van der Horst, 2000; Van der Horst et al., 2002). In several aspects, Lp is

comparable to low-density lipoprotein (LDL), the predominant transporter of cholesterol in mammals. LDL comprises a single non-exchangeable apolipoprotein, apoB, that is produced by the liver as a large, monomeric protein of 4536 amino acids (for review see Shelness and Sellers, 2001). The protein component of Lp also consists of non-exchangeable apolipoprotein, apolipophorin (apoLp)-I and apoLp-II, which are derived through post-translational cleavage from a common precursor protein of 3359 amino acids, synthesized in the fat body (Weers et al., 1993; Bogerd et al., 2000). Sequence and domain structure analysis indicate that this insect precursor protein and apoB are homologous and have emerged from an ancestral gene (Babin et al., 1999; Mann et al., 1999; Segrest et al., 2001).

Uptake of LDL is mediated by the LDL receptor (LDLR), which is the prototype for a large class of endocytic transmembrane receptors (for reviews see Brown et al., 1997; Hussain et al., 1999). Endocytosis of LDL has been extensively investigated and shown to result in the degradation of the complete lipoprotein particle in lysosomes (for reviews see Goldstein et al., 1985; Brown and Goldstein, 1986). Recently, a receptor expressed by the fat body of *Locusta migratoria* has been cloned and sequenced, and identified as a novel member of the LDLR family (Dantuma et al., 1999). This insect lipophorin receptor (LpR) was shown to mediate endocytic uptake of Lp in transiently-transfected COS-7 cells. A characteristic feature of Lp is its functioning as a reusable shuttle both at rest and during flight activity. Thus, the particle selectively loads and unloads lipids at target tissues, without concomitant degradation of Lp (for reviews see Van der Horst, 1990; Soulages and Wells, 1994; Ryan and Van der Horst, 2000; Van der Horst et al., 2001, 2002). In apparent contrast to the concept of selective lipid uptake, however, during developmental stages of larval and young adult locusts, receptor-mediated endocytic uptake of Lp in the fat body was demonstrated (Dantuma et al., 1997). These authors additionally showed that incubation of fat body tissue with Lp resulted in uptake of lipids, however, without substantial degradation of the apolipoprotein component. The involvement of an LDLR family member in lipoprotein metabolism implies complete lysosomal degradation of Lp, which is in disagreement with these findings. Thus far, the intracellular distribution after internalization of Lp mediated by LpR had not been investigated. Therefore, the intriguing question remained to be answered whether this novel LpR, in contrast to all other LDLR family members, is able to recycle its ligand after internalization.

LDL, along with di-ferric transferrin (Tf), has been extensively used to study intracellular transport of ligands that are internalized by receptor-mediated endocytosis (for reviews see Goldstein et al., 1985; Brown and Goldstein, 1986; Mellman, 1996; Mukherjee et al., 1997). Via clathrin-coated pits, the receptor-ligand complexes enter the cell in vesicles that subsequently fuse with tubulo-vesicular sorting endosomes. Due to mild acidification of the vesicle lumen, LDL dissociates from its receptor, but Tf merely unloads its two iron-ions and remains attached to the Tf receptor (TfR) (Mellman, 1996; Mukherjee et al., 1997). After repeated fusions with endocytic vesicles, sorting endosomes become inaccessible to newly internalized material. Whereas the released LDL particles are retained in the sorting endosome, most of the remaining membrane constituents (e.g. LDLR and TfR), enter the tubular extensions (Dunn and Maxfield, 1989). The tubules bud off and are delivered to the morphologically distinct endocytic recycling compartment (ERC) (Yamashiro et al., 1984; Mayor et al., 1993). Consequently, Tf accumulates in these large, long-lived, juxtanuclear organelles and, eventually, exits the compartments with a $t_{½}$ of ~7 min

(Mayor et al., 1993; Ghosh et al., 1994). Sorting endosomes, however, mature into lysosomes (Dunn et al., 1989; Stoorvogel et al., 1991) in which LDL particles are completely degraded (for reviews see Goldstein et al., 1985; Brown and Goldstein, 1986).

In the present study, CHO cell lines, in which the intracellular LDL and Tf transport pathways are well characterized, were stably transfected with LpR cDNA. These transfected cells were used to analyze the distribution and sorting of internalized insect and mammalian ligands, simultaneously. Multicolor imaging allowed visualization of multiple fluorescently-labeled ligands after endocytic uptake with high temporal and spatial resolution. Incubation of LpR-transfected CHO cells with Lp in combination with either LDL or Tf initially resulted in colocalization of the insect lipoprotein with LDL in sorting endosomes. However, in contrast to LDL that dissociates from its receptor, Lp is efficiently removed from these vesicles and, together with LpR, accumulates in the Tf-positive ERC, as confirmed with immunofluorescence. In addition to Lp, LpR is capable of binding and internalizing human receptor-associated protein (RAP), a ligand that is structurally unrelated to lipoproteins. Like Lp, this ligand is transported to the ERC after receptor-mediated endocytosis. Similar to Tf, internalized Lp is resecreted from the cells with a $t_{½}$ of ~13 min and thereby escapes the lysosomal fate of endocytosed LDL particles. This provides the first example of an LDLR homologue that, in contrast to all the other family members, is able to recycle LDL-like lipoprotein upon receptor-mediated endocytosis.

Materials and methods

Antibodies, reagents and proteins

Polyclonal rabbit-anti-LpR 9218 antibody was raised against a synthetic peptide representing the C-terminal 19 amino acids (865-883) of LpR (Dantuma et al., 1999), polyclonal rabbit-anti-apoLp-I and apoLp-II antibodies were raised against the apolipophorins as described by Schulz et al. (1987); rabbit-anti-LDLR 121 antibody was a generous gift from Dr. Ineke Braakman (Department of Bio-Organic Chemistry, Utrecht University, Utrecht, The Netherlands) human RAP was a generous gift from Dr. Michael Etzerodt (IMSB, Aarhus University, Aarhus, Denmark) and LysoTracker Yellow (Molecular Probes) was a generous gift from Arjan de Brouwer (Department of Biochemistry of Lipids, Utrecht University, Utrecht, the Netherlands). Geneticin (G-418) (GibcoBRL), precision protein standards prestained broad range marker (Bio-Rad), alkaline phosphatase-conjugated affinipure goat-anti-rabbit IgG (AP-GAR) and Cy5-conjugated goat-anti-rabbit IgG (Cy5-GAR) (Jackson ImmunoResearch Laboratories Inc.), leupeptin, aprotinin, monensin and nocodazole (Sigma), DiI(C_{18}(3)) (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine percholate), Oregon Green 488 carboxylic acid and tetramethylrhodamine-labeled Tf (Molecular Probes), saponin and iodine monochloride (ICN Biochemicals), BSA and cold water fish gelatin (Sigma), ^{125}I[iodine] (3.9 GBq/ml; Amersham Pharmacia Biochem) and chloramine-T (Merck) were obtained from commercial sources. Human LDL was isolated from blood plasma (Bloedbank Midden Nederland) as described by Redgrave et al. (1975); Lp was isolated from locust hemolymph by ultracentrifugation (Dantuma et al., 1996) with the following modifications to the original protocol. Hemolymph collected from 12-15 days

old locusts that were reared under crowded conditions was immediately diluted in ice-cold insect saline buffer supplemented with leupeptin (4 μg/ml) and aprotinin (4 μg/ml).

Cell culture

CHO cells were cultured in 75 cm^2 polystyrene culture flasks (Nunc Brand Products) with growth medium containing HAM's F-10 Nutrient mixture, 10% heat inactivated fetal bovine serum, 100 U/ml penicillin G sodium and 100 μg/ml streptomycin sulfate in 85% saline (Gibco BRL) at 37°C in a humidified atmosphere of 5% CO_2. Growth medium of LpR-expressing cells was supplemented with 300 μg/ml G-418. For fluorescence microscopy and confocal laser scanning microscopy, cells were grown on 15 or 18-mm and 24-mm glass cover slips (Menzel-Gläser) in 12-wells (3.5 cm^2/well) and 6-wells (9.6 cm^2/well) multidishes (Nunc Brand Products), respectively.

Generation of CHO cell lines stably expressing LpR

Wild-type CHO cells were grown to ~40% confluency in 6-wells multidishes and transfected for 20 h with 5 μg of piLR-e plasmid (Dantuma et al., 1999) DNA in 2 ml serum free growth medium supplemented with 20 μl Lipofectin reagent (Invitrogen Life Technologies) according to the supplier's protocol. The cells were grown for 7-10 days in selective growth medium, containing 400 μg/ml G-418, to obtain stably transfected cells. These cells were isolated by limited dilution to generate monoclonal cell lines and checked for LpR expression. Because variable levels of LpR expression were observed in the different cell lines, we used a monoclonal CHO(LpR) cell line that showed the highest expression level of LpR for the incubation experiments described in this study.

Western blot analysis of CHO cell membrane extracts

Cells were harvested from 75 cm^2 polystyrene flasks at ~80% confluency, resuspended in CHAPS buffer (20 mM HEPES, 124 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 2.5 mM Na_2HPO_4, 1.2 mM $MgSO_4$, 1 mM EDTA, 0.1 mM benzamidine, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1% CHAPS), incubated for 10 min on ice, and spun down at 15000 *g* for 10 min at 4°C. Supernatant was diluted with 1 volume glycerol and soluble membrane proteins were either heated for 5 min at 95°C in Laemmli buffer (Laemmli, 1970) or immediately dissolved in Laemmli buffer with 0.025% SDS and no reducing agents prior to separation by SDS-PAGE on a 10% polyacrylamide gel. The separated membrane proteins were transferred to polyvinylidene fluoride membrane (Millipore) by Western blotting and incubated with rabbit-anti-LpR (1:2000) or rabbit-anti-LDLR antibodies (1:5000) as indicated for 2 h, followed by 1 h AP-GAR incubation. Hybridized AP-GAR was visualized by incubating the blot in TSM buffer, containing 100 mM Tris-HCl, 100 mM NaCl, 10 mM $MgAc_2$, 50 μg/ml p-nitro blue tetrazolium chloride (NBT; Boehringer Mannheim), 25 μg/ml 5-bromo-4-chloro-3-indoyl-phosphate p-toluidine (BCIP; Roche Diagnostics), pH 9.0.

Incubation of CHO cells with fluorescently-labeled ligands

LDL and Lp (1 mg/ml) were fluorescently labeled in PBS with 50 μl/ml DiI in DMSO (3 μg/μl) at 37°C under continuous stirring for 16 h and 3 h, respectively. Lp and RAP (1 mg/ml) were labeled with 20 μl/ml OG dissolved in DMSO (1 μg/μl) at room temperature under continuous stirring for 1 h according to the manufacturer's instructions. Fluorescently-labeled lipoproteins were purified with Sephadex G-25

PD-10 columns (Amersham Pharmacia Biotech) to replace the PBS by incubation medium containing 10 mM HEPES, 50 mM NaCl, 10 mM KCl, 5 mM $CaCl_2$, 2 mM $MgSO_4$, pH 7.4. OG-RAP was dialyzed against incubation medium using standard cellulose membrane (Medicell International). For endocytic uptake, CHO cells were incubated with 10 μg/ml DiI-LDL, 25 μg/ml OG-Lp, 3.6 μg/ml OG-RAP and 25 μg/ml TMR-Tf as indicated for 15 min at 37°C or 30 min at 18°C. Cells were rinsed in incubation medium and either immediately fixated in 4% paraformaldehyde diluted in PBS for 30 min at room temperature, or chased in growth medium at 37°C for variable time periods. When indicated, nocodazole (5 μM) or monensin (25 μM) was added to the medium prior to, as well as during the chase.

Immunofluorescence

Fixated cells were washed twice with PBS buffer and permeabilized with PBS buffer supplemented with 1.0 mg/ml saponin (PBSS) for 5 min at room temperature. The cells were subsequently incubated with PBSS containing 50 mM glycin for 10 min and 5% BSA for 30 min at room temperature. The cells were blocked twice for 5 min with 0.1% cold water fish gelatin in PBSS (PBSSG) at room temperature and incubated with corresponding primary antibodies (1:500) for 1 h at 37°C. After rinsing 4 times for 5 min with PBSSG at room temperature, the samples were processed for indirect immunofluorescence by incubation with Cy5-GAR for 30 min at 37°C and rinsed an additional 4 times with PBSSG.

Microscopy and image processing

Cover slips with fixated cells were mounted in Mowiol supplemented with anti-fade reagent (DABCO) and examined on a fluorescence Axioscop microscope (Zeiss) with a Hg HBO-50 lamp and a Plan-Neofluar 100×/1.30 oil lens. Using FITC/TRITC filters, digital images were acquired with a DXM 1200 digital camera and ACT-1 version 2.00 software (Nikon Corporation).

To image living cells, we mounted the coverslips in a temperature-controlled aluminium chamber and incubated the cells at 37°C in growth medium supplemented with 1 μl/ml 1 mM LT, where indicated. Confocal multicolor images of cells were acquired using a Leica TCS-NT confocal laser scanning-system on an inverted microscope DMIRBE (Leica Microsystems) with a PL APO 40×/1.25-0.75 oil lens (Leica Microsystems) and an argon-krypton laser as excitation source. Emission of OG, excited with the 488 laser line, was detected using a 530/30 nm (RSP 580) bandpass filter. DiI, TMR and LT were excited with the 568 nm laser line and detected using a 600/30 nm (RSP 660) bandpass filter. The 647 nm laser line was used to excite Cy5 and emission was detected with a 665 nm longpass filter.

Images were processed using Scion Image beta version 4.0.2 (Scion Corporation) and PaintShop pro 7.00 (Jasc Software) software. SigmaPlot for Windows 4.00 (SPSS Inc) was used to generate surface fluorescence intensity mesh plots. To quantify the relative intensity of fluorescently-labeled ligand in cells, the average brightness of pixels in manually defined areas covering the cells was determined using the Scion Image software. The digital data of more than 200 individual cells per data point were processed using Microsoft Excel 2000 (Microsoft Corporation) and plotted using the SigmaPlot software.

Incubation of CHO cells with ^{125}I-labeled ligands

Lp was labeled with ^{125}I[iodine] using iodine monochloride according to McFarlane (1958), resulting in a specific labeling activity of 85 and 236 cpm/ng Lp. ^{125}I-RAP was prepared using chloramine-T according to Rodenburg et al. (1998), resulting in a specific labeling activity of ~45000 cpm/ng protein. Two experiments were performed in duplicate, using wild-type CHO and CHO(LpR) cells that were cultured in 12-well plates and grown to ~70% confluency. The cells were incubated for 45 min at 37°C in incubation medium containing 25 µg/ml ^{125}I-Lp or 83 ng/ml (2.1 nM) ^{125}I-RAP without monensin, followed by an additional 15 min in the presence of 25µM monensin. The cells were placed on ice, washed twice with cold wash buffer, containing 150 mM NaCl, 50 mM Tris-HCl, 2% BSA, pH 7.4, and subsequently lysed and dissolved in 0.1 N NaOH. The radioactivity of samples was determined with a Tri Carb 2300 TR liquid scintillation analyzer (Packard) in Emulsifier Safe liquid scintillation fluid (Packard) and a maximal counting time of 10 min per sample. To determine the total cell protein per well, cells were washed thrice with 4°C HEPES buffer and incubated for 4 h at 4°C in a lysis buffer, containing 50 mM Tris-HCl (pH 7.7), 150 mM NaCl, 0.1 mM benzamidine, 1 mg/ml leupeptin, 1 mg/ml aprotinin, and 1% NP40. Protein concentrations were determined using the colorimetric detergent compatible protein assay (Bio-Rad).

Results

Expression of LpR by stably transfected CHO cells

In order study the endocytic capacity of LpR *in vitro*, LDLR-deficient CHO cells (*ldlA* cells; Kingsley and Krieger, 1984) that produce intracellular nonfunctional LDLR intermediates were stably transfected with the mammalian expression vector pcDNA3, harboring the full-length LpR cDNA [*ldlA*(LpR); Dantuma et al., 1999]. Additionally, wild-type CHO cells were stably transfected with the same construct [CHO(LpR)] to be able to compare the intracellular pathways of internalized mammalian and insect lipoproteins, simultaneously. The expression of LpR by both transfected cell lines was analyzed using detergent cell extracts that were separated by SDS-PAGE under reducing and non-reducing conditions. The proteins were transferred to polyvinylidene fluoride membrane and immunoblotted with polyclonal anti-LpR rabbit antibody raised against the cytoplasmic tail of LpR. These Western blots showed a similar LpR expression level of both transfected CHO cell lines (Fig. 1A and B). Under reducing conditions (Fig. 1A), the apparent molecular weight of LpR increased from ~120 kDa (Fig. 1B, non-reducing conditions) to ~150 kDa (Fig. 1A, reducing conditions), which is consistent with the reduction of multiple disulfide bonds present in the cysteine class A repeats and the EGF precursor homology domain. Moreover, the results demonstrate that LpR is expressed as a receptor with a molecular weight of ~150 kDa (Fig. 1A, reducing conditions), which is higher than the predicted 98 kDa (Dantuma et al., 1999). This suggests that the receptor is glycosylated, like all the other members of the LDLR family (Russell et al., 1984). The endogenous LDLR expression of CHO cells was unaffected by transfection with LpR cDNA, as assessed from Western blot analysis using the polyclonal anti-LDLR rabbit antibody raised against the extracellular domain of LDLR (Fig. 1C and D).

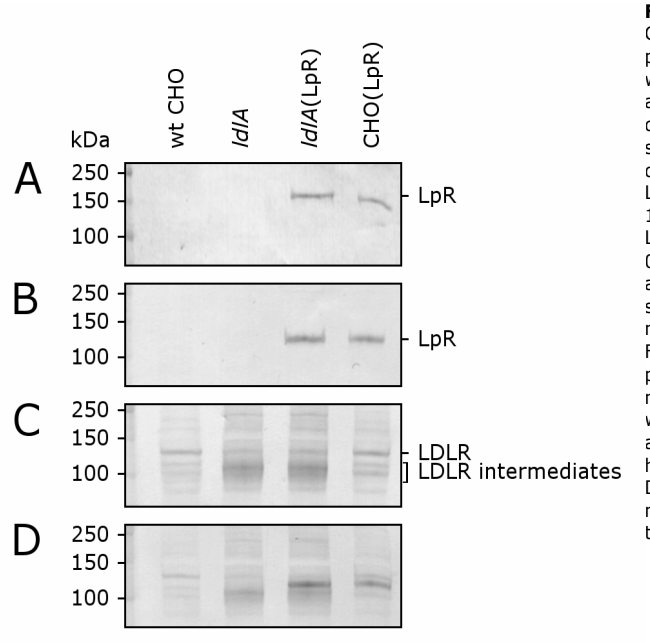

Figure 1. LpR expression in CHO cell lines. Membrane proteins were isolated from wild-type CHO, *ldlA*, *ldlA*(LpR) and CHO(LpR) cells as described in the methods section. Samples were either denatured for 5 min at 95°C in Laemmli buffer (Laemmli, 1970) (A), or dissolved in Laemmli buffer containing 0.025% SDS and no reducing agents, and immediately subjected to SDS-PAGE under non-reducing conditions (B-D). Following transfer to polyvinylidene fluoride membrane, LpR was detected with anti-LpR antibody (A, B, and D) and LDLR with anti-human LDLR antibody (C and D). The molecular weight markers (kDa) are indicated on the left of each panel.

LpR mediates uptake of Lp and human RAP in stably transfected CHO cells

To investigate the functional ligand-binding specificity of LpR and LDLR, LpR-transfected cells were incubated with fluorescently-labeled ligands in a buffer that was supplemented with HEPES (i.e. incubation medium) to retard the transit of internalized ligands at the early endosomal stage (Sullivan et al., 1987). Upon 15 min of incubation at 37°C with DiI-labeled human LDL (DiI-LDL), numerous cytoplasmic vesicles distributed throughout CHO(LpR) cells could be observed (Fig. 2A). Such a punctate staining pattern, indicative for receptor-mediated endocytosis, was absent in *ldlA*(LpR) cells (Fig. 2B). This indicates that LDL uptake is exclusively accomplished by the endogenous LDLR, and not a result of aspecific endocytosis via LpR. A comparable particulate pattern was observed in LpR-transfected cells incubated with DiI-labeled Lp (DiI-Lp) (Fig. 2C), however, not in non-transfected cells (Fig. 2D). DiI is a fluorescent lipid homologue that incorporates in the lipid moiety of lipoproteins. To confirm the concomitant endocytic uptake of the protein component of the lipoprotein, Lp was labeled covalently with the amine-reactive fluorescent probe Oregon Green 488 (OG). Analogous incubation experiments with OG-labeled Lp (OG-Lp) led to a similar endocytic uptake as could be visualized by DiI-Lp (Fig. 2E and F). These data suggest that the lipid uptake mediated by LpR is a result of Lp internalization rather than a selective lipid-transfer mechanism occurring at the cell surface. To verify that the internalized lipoproteins are localized in endosomes after a 15 min incubation period at 37°C, the uptake experiments were repeated for 30 min at 18°C. Intracellular distribution of endocytosed ligands stagnates at a temperature of 18°C or below, preventing lysosomal degradation of ligands and recycling of receptors (Sullivan et al., 1987). The endocytic vesicle patterns of CHO(LpR) cells incubated at either

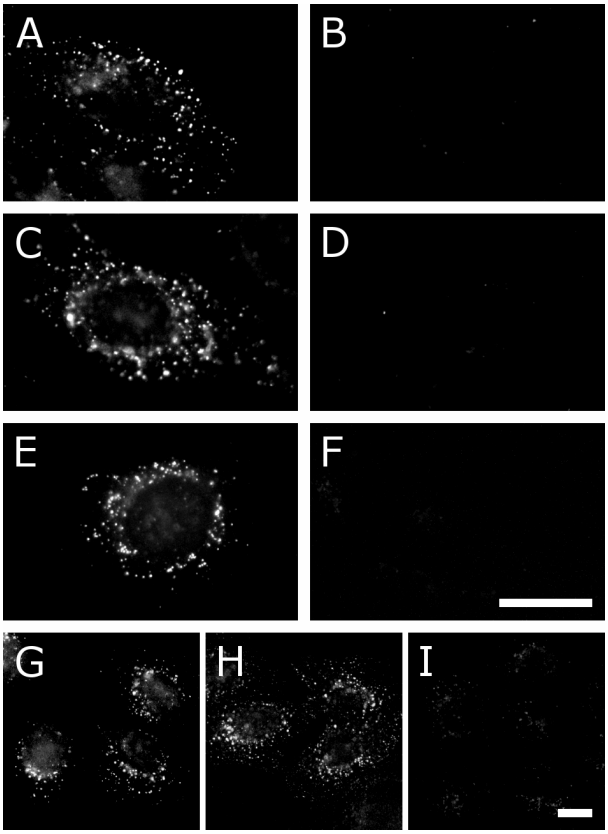

Figure 2. Receptor-mediated endocytic uptake of fluorescently-labeled lipoproteins by CHO cells. CHO cells were incubated with fluorescently-labeled lipoproteins in incubation medium for 15 min at 37°C, fixated with paraformaldehyde and mounted in mowiol (A-F). Single cells were imaged using fluorescence microscopy to visualize the accumulation of fluorescently-labeled ligands in endocytic vesicles. CHO(LpR) (A) and *ldlA*(LpR) (B) cells were incubated with DiI-LDL. Lp labeled with DiI (C and D) or OG (E and F) was used to incubate CHO(LpR) (C and E) and wild-type CHO (D and F) cells. Uptake of fluorescently-labeled Lp is reduced by an excess of unlabeled Lp. CHO(LpR) cells were also incubated for 15 min at 37°C (G) or 30 min at 18°C (H and I) in incubation medium containing OG-Lp in the absence (G and H) or presence (I) of a tenfold excess of unlabeled Lp. Bars, 20 μm (F) and 10 μm (I).

temperature were indistinguishable (Fig. 2G and H), which strongly suggests that Lp is transferred to sorting endosomes after receptor-mediated endocytosis. Uptake of fluorescently-labeled Lp could be reduced with an equimolar concentration, and almost completely inhibited with a tenfold excess of unlabeled Lp (Fig. 2I). This indicates that labeled and unlabeled Lp compete for the same binding site. Therefore, it is most unlikely that the interaction between Lp and LpR is altered by the covalently-bound OG label. From these experiments, we conclude that LDL uptake is restricted to endogenous LDLR-expressing cells and that Lp uptake is exclusively mediated by LpR.

RAP has been shown to inhibit the binding of lipoproteins to LDLR family members, such as LDLR-related protein (LRP), very low-density lipoprotein receptor (VLDLR) and megalin (Herz et al., 1991; Kounnas et al., 1992; Battey et al., 1994), but has only weak affinity for LDLR itself (Medh et al., 1995). RAP serves as a molecular chaperone to assist the folding of several LDLR family members and prevents premature ligand interaction in the endoplasmic reticulum (for reviews see Bu and Schwartz, 1998; Bu and Marzolo, 2000). As expected, when CHO(LpR) cells were incubated with DiI-LDL and an equimolar concentration of human RAP, endocytosis of LDL was not significantly reduced (Fig. 3A). However, endocytic uptake of Lp could be completely prevented by an equimolar concentration of RAP (Fig. 3B). Inhibition of Lp endocytosis by RAP indicates that LpR binds Lp in the prevalent lipoprotein-binding

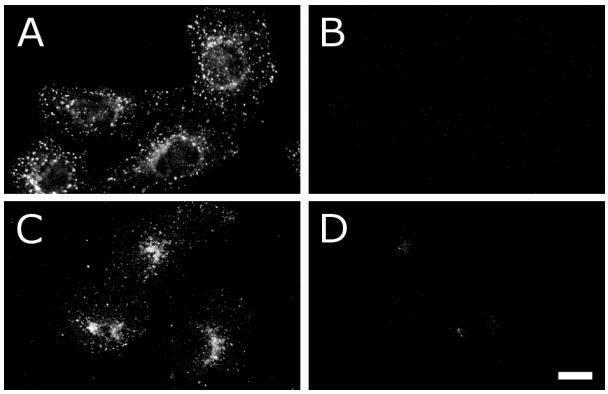

Figure 3. LDL endocytosis by CHO(LpR) cells is not significantly reduced by an equimolar concentration of RAP; however, endocytic uptake of Lp is completely inhibited. CHO(LpR) cells were incubated for 30 min at 18°C with DiI-LDL (A) or OG-Lp (B) in the presence of an equimolar concentration unlabeled RAP. RAP is internalized by LpR-expressing CHO cells. CHO(LpR) (C) and wild-type CHO (D) cells were incubated with OG-RAP for 30 min at 18°C. Bar, 10 μm.

manner, namely via its cysteine-rich ligand-binding domain (Dantuma et al., 1999). Additionally, the observation that a 1:1 ratio of RAP to OG-Lp is sufficient to completely inhibit Lp endocytosis suggests that, in comparison to Lp, RAP has a higher affinity for LpR. Moreover, these data suggest that RAP is a ligand of LpR and, thus, could also be internalized by the insect receptor. To obtain evidence for this latter issue, we incubated CHO cells with OG-labeled RAP (OG-RAP) for 30 min at 18°C which resulted in a perinuclear vesicle distribution (Fig. 3C). Although the staining pattern appeared different from that observed in CHO(LpR) cells incubated with Lp, endocytic uptake of RAP was clearly evident. Minor amounts of RAP could also be detected in endocytic vesicles of wild-type CHO cells (Fig. 3D), which is likely due to the expression of endogenous LRP and VLDLR. However, the fluorescence intensity of these vesicles was much lower in comparison to LpR-transfected cells, thus the majority of intracellular RAP in CHO(LpR) cells is endocytosed by LpR. The observation that, in addition to Lp, RAP is also a ligand of LpR is in excellent agreement with LpR being an LDLR family member.

Mammalian and insect lipoproteins follow distinct intracellular routes

Receptor-bound LDL is rapidly delivered to sorting endosomes upon endocytosis by mammalian cells (for reviews see Mellman, 1996; Mukherjee et al., 1997). The results of the incubation experiments at 18°C (Fig. 2H) suggest that Lp and LDL are internalized and transferred to the same vesicles. To investigate whether Lp accumulates in these tubulo-vesicular endosomes, CHO(LpR) cells were incubated at 18°C with OG-Lp in incubation medium supplemented with DiI-LDL. There was significant colocalization of Lp (Fig. 4A) with LDL-containing endocytic vesicles (Fig. 4B and C) that were distributed throughout the cell, which supports the assumption that Lp accumulates in sorting endosomes after endocytic uptake.

In sorting endosomes, LDL dissociates from LDLR due to mild luminal acidification after which the ligand is degraded in lysosomes. The receptor, however, is transported back to the cell surface via the ERC for additional uptake of extracellular LDL (for reviews see Mellman, 1996; Mukherjee et al., 1997). By observing living cells with confocal laser scanning microscopy, we were able to visualize the sorting of mammalian and insect lipoproteins simultaneously, immediately after endocytic uptake. CHO(LpR) cells were preincubated with OG-Lp and DiI-LDL for 15 min at 37°C

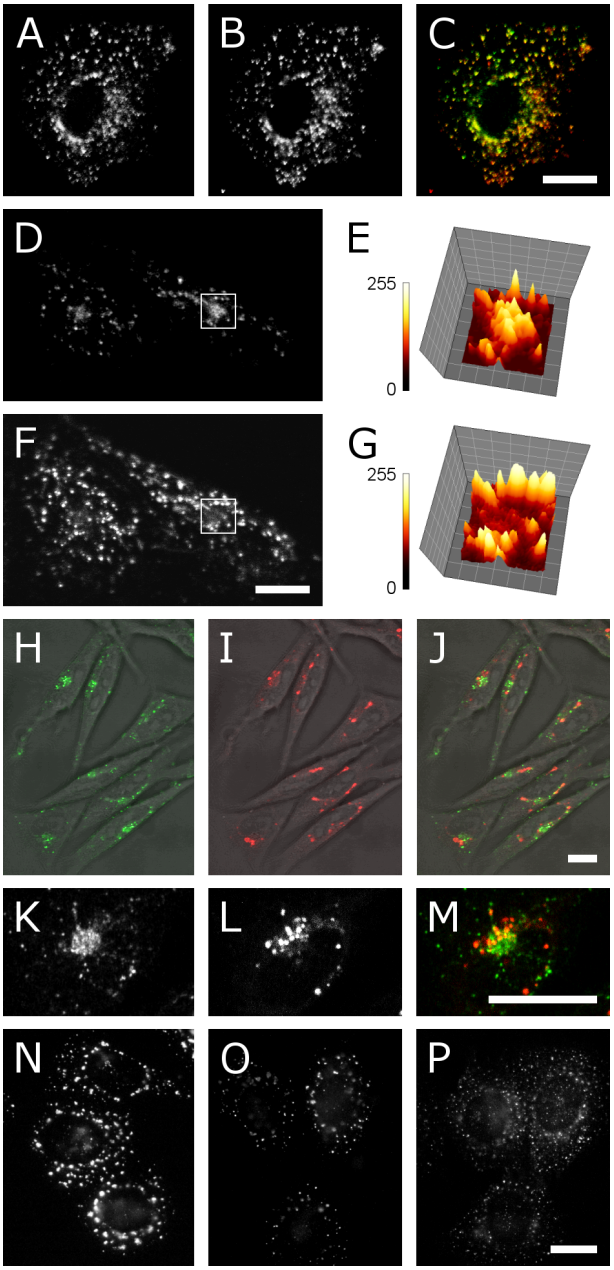

Figure 4. Lp colocalizes with LDL in early endocytic vesicles. CHO(LpR) cells were allowed to simultaneously internalize OG-Lp (A) and DiI-LDL (B) in incubation medium for 30 min at 18°C. Fixated cells were analyzed using confocal laser microscopy to visualize the colocalization of the ligands in endosomes by overlaying the two images (C). The Lp-positive juxtanuclear compartment is depleted of LDL. CHO(LpR) cells were simultaneously preincubated with OG-Lp and DiI-LDL. After the preincubation, the cells were transferred to an aluminium chamber and incubated in chase medium at 37°C. At 10 min, large amounts of Lp concentrated in the juxtanuclear region (D), whereas LDL remained spatially distributed throughout the entire cell interior (F). Within a defined area (squares in D and F), the relative fluorescent intensity of the juxtanuclear-positioned structure was plotted on a relative scale (from 0 to 255, indicated by the vertical bar) for OG-Lp (E) and DiI-LDL (G). Internalized Lp accumulates in a non-lysosomal juxtanuclear compartment. CHO cells stably expressing LpR were preincubated with OG-Lp, rinsed in HEPES buffer and mounted in an aluminium chamber. The cells were subsequently incubated at 37°C in chase medium that was supplemented with LT. Images were generated with multicolor imaging, using confocal laser microscopy to spatially visualize internalized Lp and LT, simultaneously, in living cells. After a chase of 15 min, OG-Lp-positive endocytic vesicles were highly concentrated in the juxtanuclear region (H), which was depleted of LT (I). Partial colocalization with LT was visualized by merging the two images (J). To enhance the visibility of the spatial distribution of Lp and LT, a transparent bright field image of the observed cells was overlayed with fluorescent images. Additionally, detailed images of a single juxtanuclear structure were taken to visualize the minimal colocalization (K-M). Intracellular transport of ligands by LpR is microtubule-dependent. CHO(LpR) cells were preincubated with fluorescently-labeled ligand in the presence of 5 μM nocodazole. The cells were subsequently incubated for an additional 30 min at 37°C in chase medium supplemented with 5 μM nocodazole. Fixated cells were observed with confocal laser microscopy and showed a peripheral localization of vesicles that contained LDL (N), Lp (O) or RAP (P). Lp is shown in grey (A, D, K, and O) or green (C, H, J, and M); LDL is shown in grey (B, F, and N) or red (C); LT is shown in red (I, J, and M) or grey (L); colocalization is shown in yellow (C, J, and M). Bars, 20 μm (C, F, J, M, and P).

and subjected to a chase in growth medium without fluorescently-labeled ligands (chase medium) for an additional 30 min at 37°C. Within 10 min, a large amount of Lp concentrated in the juxtanuclear area (Fig. 4D and E) in which LDL was almost completely absent (Fig. 4F and G). To investigate whether these vesicles were late endosomes or lysosomes, the membrane permeable probe, LysoTracker Yellow (LT), a weakly basic amine that selectively accumulates in cellular compartments with low luminal pH (i.e. lysosomes; Griffiths et al., 1988), was added to the chase medium (Fig. 4H and I). As shown in Fig. 4J, there was almost no colocalization of Lp with LT, and in areas where there was apparent overlap, the size and shape of the structures appeared different (Fig. 4K-M). This result implies that Lp is not destined to be degraded via the classic LDL pathway. Altogether, these results confirm that, in contrast to LDL, internalized Lp is not destined for lysosomal degradation.

The microtubule-depolymerising agent nocodazole was used to investigate whether this perinuclear targeting was microtubule dependent. Depolymerization of microtubules has little effect on endocytosis, however, microtubule-dependent transport of internalized material is inhibited (Jin and Snider, 1993). Incubation of CHO(LpR) cells with DiI-LDL and OG-Lp in the presence of 5 μM nocodazole followed by a chase for 30 min with an equal concentration of nocodazole resulted in the formation of enlarged LDL-labeled vesicles that were located peripherally in the cells (Fig. 4N). A similar distribution of endocytic vesicles was observed when Lp or RAP was used (Fig. 4O and P, respectively). Although vesicles containing Lp or RAP appeared smaller in size, and their fluorescence intensity less in comparison to LDL-containing vesicles, these data indicate that transit of LpR-bound ligands (i.e. Lp and RAP) is microtubule dependent.

Lp and RAP are transported to the ERC by LpR

To determine whether Lp is translocated to the juxtanuclear localized ERC, we used Tf which converges in the ERC after endocytic uptake due to the durable association with TfR (Yamashiro et al., 1984; Mayor et al., 1993). CHO(LpR) cells that were incubated with OG-Lp and tetramethylrhodamine-labeled Tf (TMR-Tf), and subjected to a chase, show that Lp is translocated to the ERC within 10 min (Fig. 5, left panel). Despite a small portion of individual vesicles that remained dispersed throughout the cell, the majority of Lp colocalized with Tf in the ERC (Fig. 5, middle and right panel) from where molecules eventually exit the cell (Yamashiro et al., 1984). However, the convergence of Lp in the ERC appeared slightly slower in comparison to the rapid transport of Tf, which is most likely the result of different sorting rates.

Additional evidence for the transport of Lp to the ERC was obtained from experiments with monensin, a carboxylic ionophore which disrupts the route of recycling receptors (e.g. LDLR and TfR) by preventing the receptors from returning to the cell surface and thereby causing them to reside within the ERC (Basu et al., 1981; Stein et al., 1984). A concentration of 25 μM monensin appeared sufficient to interrupt receptor recycling and trap internalized receptors of CHO(LpR) cells that were preincubated with DiI-LDL or OG-Lp, and chased for an additional 30 min. Monensin did not significantly affect lysosomal targeting of LDL (Fig. 6A), however, Lp accumulated in the juxtanuclear area (Fig. 6B). OG fluorescence observed in the ERC represents either undegraded OG-Lp or OG released from degraded OG-Lp. To confirm the concomitant transport of the non-exchangeable apolipoprotein matrix of Lp with the

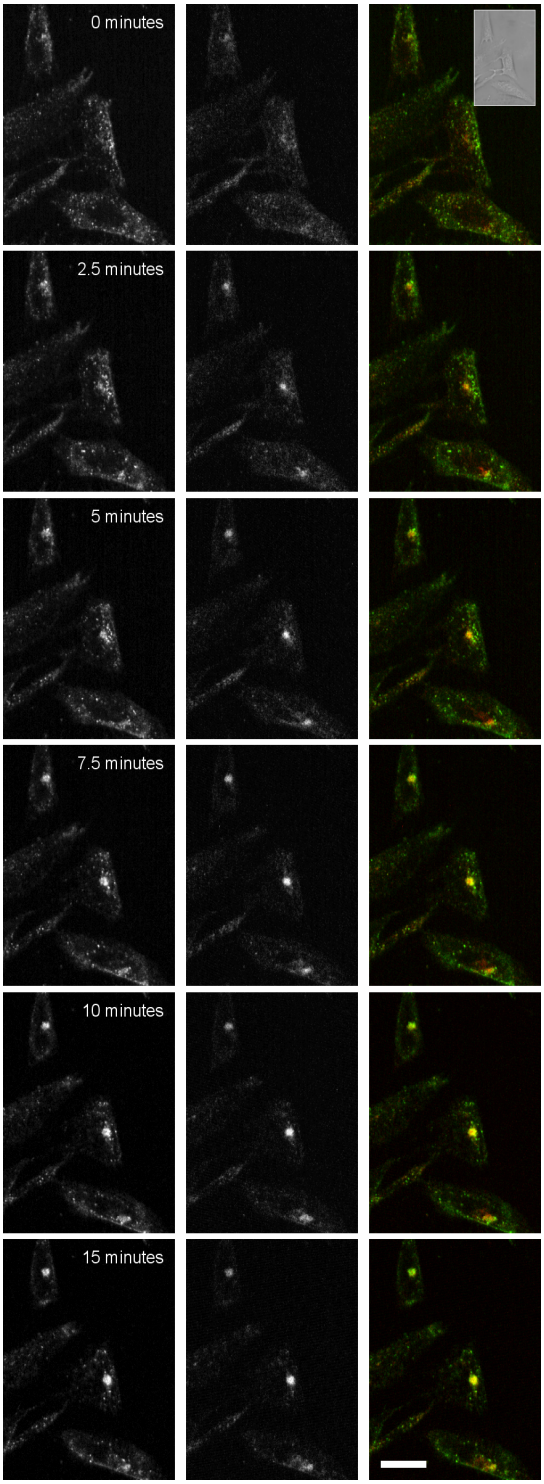

Figure 5. Lp colocalizes with internalized Tf in the ERC. Immediately after preincubation for 20 min at 18°C with OG-Lp and TMR-Tf, LpR-transfected CHO cells were rinsed in HEPES buffer and mounted in an aluminium chamber. The living cells were observed at 37°C in chase medium and imaged using confocal laser microscopy. Digital multicolor images of OG-Lp (left panel; green, shown in grey) and TMR-Tf (middle panel; red, shown in grey) were taken at defined time points as indicated in the left panel. Colocalization was visualized by merging the two images of the same time point (right panel; shown in color). Colocalization is shown in yellow. The insert in the upper right panel shows a bright field image of the observed cells immediately after preincubation. Bar, 20 μm.

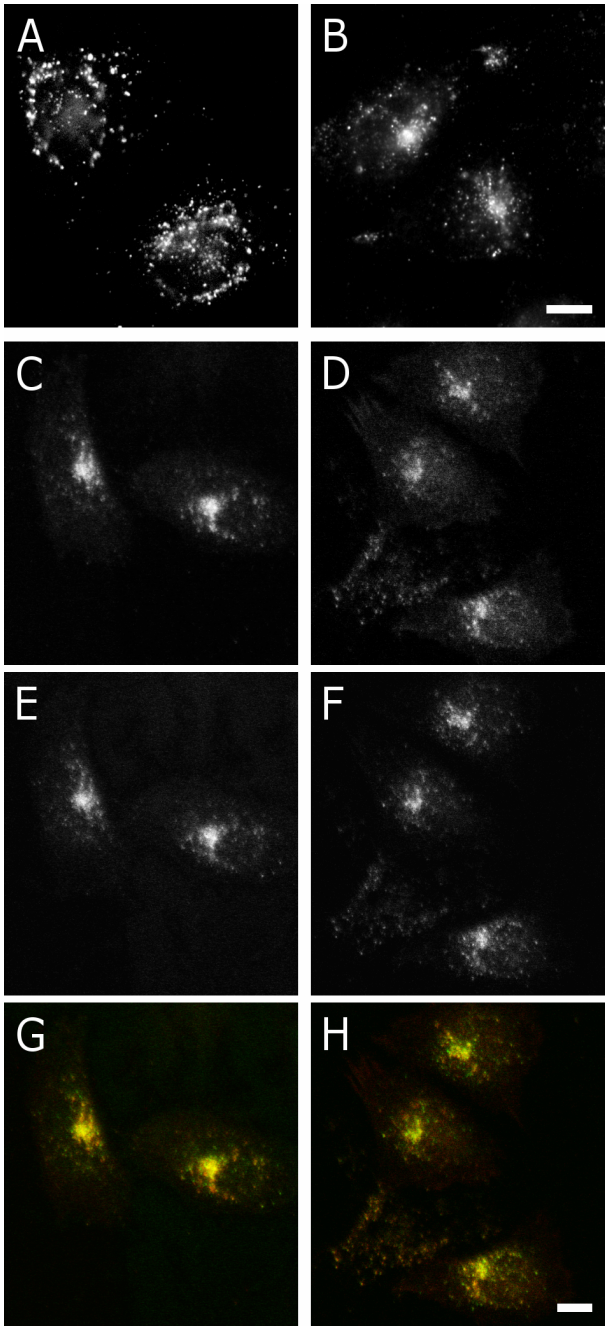

Figure 6. Internalized Lp is trapped intracellularly by monensin. CHO(LpR) cells were incubated with fluorescently-labeled ligands in the presence of 25 µM monensin for 30 min at 18°C. The cells were subsequently chased for 30 min at 37°C with an equal concentration of monensin and mounted in mowiol after fixation. LDL was scattered throughout the cell in vesicles (A), however, Lp was predominantly localized in the juxtanuclear area (B). The non-exchangeable protein matrix of Lp is transported to the ERC. CHO(LpR) cells were allowed to take up OG-Lp for 15 min at 37°C in the presence of 25 µM monensin. After a chase of 30 min with an equal concentration of monensin, the cells were washed and labeled with antibodies against apoLp-I and apoLp-II which were visualized with a Cy5-labeled second antibody. ApoLp-I (C) and apoLp-II (D) colocalized with the fluorescent label OG that represents intracellular Lp (E and F) in the juxtanuclear area (G and H). LDL is shown in grey (A); Lp is shown in grey (B, E, and F) or green (G and H); apoLp-I is shown in grey (C) or red (G); apoLp-II is shown in grey (D) or red (H); colocalization is shown in yellow (G and H). Bars, 10 µm (B and H).

fluorescent label OG to the ERC, we used antibodies against apoLp-I and apoLp-II to immunolocalize the proteins. Cells were fixated after preincubation with OG-Lp and a chase of 30 min in the presence of monensin. The cells were subsequently incubated

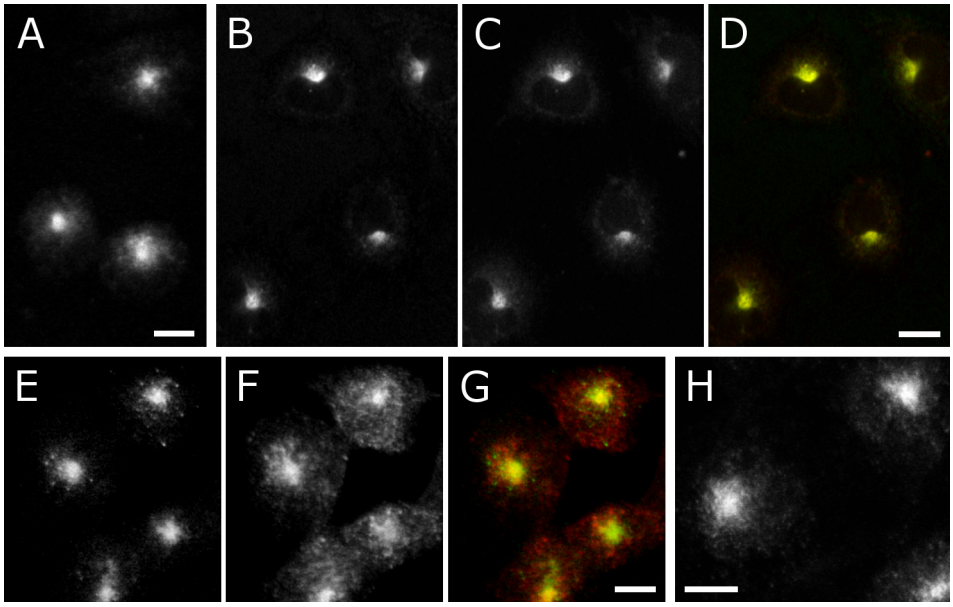

Figure 7. Internalized RAP accumulates in the juxtanuclear area. CHO(LpR) cells preincubated with OG-RAP and chased for 30 min at 37°C in the presence of 25 μM monensin were fixated and mounted in mowiol. The cells were observed with fluorescence microscopy to visualize RAP which was predominantly localized in the juxtanuclear region (A). RAP follows a transferrin-like intracellular pathway. CHO(LpR) cells were simultaneously preincubated with OG-RAP and TMR-Tf and chased for 30 min in the presence of 25 μM monensin. Digital images of fixated cells containing RAP (B) and Tf (C) were generated with cfocal laser microscopy and colocalization in the juxtanuclear area was visualized by merging the two images (D). Lp colocalizes with LpR in the ERC. To determine the location of LpR after preincubation with OG-Lp and chase for 30 min in the presence of 25 μM monensin, CHO(LpR) cells were fixated and labeled with antibodies against LpR which were visualized with a Cy5-labeled second antibody. OG-Lp (E) and LpR (F) show significant overlap in the ERC (G). LpR is also abundantly located in the ERC in the absence of ligand or monensin. CHO(LpR) cells were fixated after treatment with incubation medium for 15 min at 37°C and LpR was visualized as described above (H). RAP is shown in grey (A and B) or green (D); Tf is shown in grey (C) or red (D); Lp is shown in grey (E) or green (G); LpR is shown in grey (F and H) or red (G); colocalization is shown in yellow (D and G). Bars, 10 μm (A, D, G, and H).

with anti-apoLp-I or apoLp-II rabbit antibodies (Schulz et al., 1987) which were visualized with a Cy5-labeled goat-anti-rabbit second antibody. Both apoLp-I (Fig. 6C) and apoLp-II (Fig. 6D) were predominantly localized in the ERC and show significant overlap with OG (Fig. 6E-H). We interpret these data to indicate that the complete non-exchangeable protein matrix of Lp, comprising apoLp-I and apoLp-II, is transported to the ERC.

Above we showed that LpR is capable of binding and internalizing human RAP (Fig. 3C). To investigate whether endocytosed RAP is also transported to the ERC, we repeated the incubation experiments with monensin using RAP. Subjecting CHO(LpR) cells to a chase after preincubation with OG-RAP in the presence of monensin resulted in the convergence of RAP in a single spot near the nucleus (Fig. 7A). When TMR-Tf was used in combination with OG-RAP, there was significant colocalization of RAP and Tf in the ERC (Fig. 7B-D). This implies that the pathways of ligands that are internalized by LpR are determined by the intracellular route of the receptor. To

visualize the intracellular location of LpR, we used anti-LpR antibody and the Cy5-labeled second antibody to detect LpR in fixated CHO(LpR) cells. Preincubation of these cells with OG-Lp followed by a chase in medium containing monensin shows that the ligand is localized in the ERC (Fig. 7E), the organelle in which LpR is also located (Fig. 7F and G). Even in the absence of ligand or monensin, the receptor was predominantly present in the ERC (Fig. 7H), suggesting constitutive recycling of LpR without antecedent ligand binding as observed for LDLR (Anderson et al., 1982; Brown et al., 1982) and TfR (Stein and Sussman, 1986).

To quantify LpR-specific uptake, and subsequent transfer to the ERC of Lp and RAP, we incubated wild-type CHO and CHO(LpR) cells with ^{125}I-labeled Lp and RAP in the presence of monensin. Cells were preincubated with the ^{125}I-labeled ligands for 45 min at 37°C without monensin, followed by a shorter second incubation of 15 min at 37°C with the ^{125}I-labeled ligands in the presence of 25 μM monensin. These experiments revealed an LpR-mediated Lp uptake of 112 ng/mg cell protein (means of two duplo experiments, s.e.m. ± 27), which corresponds to ~350 pmol/mg cell protein. LpR-specific uptake of RAP was also determined and appeared to be 61.3 ng/mg cell protein (mean of duplo experiment, s.e.m. ± 0.44), the equivalent of ~1570 pmol/mg cell protein. This ~4.5-fold higher uptake of RAP in comparison to Lp is in good agreement with the observation that a 1:1 ratio of RAP to OG-Lp is sufficient to completely inhibit Lp endocytosis (Fig. 3B). Moreover, it supports the relatively higher affinity of RAP for LpR in comparison to that of Lp, as suggested above.

Lp resecreted from CHO(LpR) cells with a $t_{½}$ of ~13 min

Convergence of Lp and RAP in the ERC implies that these ligands are eventually resecreted into the medium (Yamashiro et al., 1984). Quantitative fluorescence microscopy was used to determine the exit rate of intracellular LDL, Lp and RAP. First, CHO(LpR) cells were analyzed after a preincubation of OG-Lp and DiI-LDL to label the endocytic pathway. Shortly after initiating the chase, the clearly visible ERC predominantly contained Lp, in which no significant amount of LDL could be detected (Fig. 8A). In contrast, the spatially distributed vesicles that were numerously present contained mainly LDL, some of which harboring only a minor amount of Lp. During the chase, the relative fluorescent intensity of OG-Lp in the ERC decreased dramatically compared to that of the individual, LDL-containing vesicles (Fig. 8B-F). Total intracellular fluorescence of DiI-LDL, OG-Lp and OG-RAP in cells that were fixated after a chase at defined time points were determined (Fig. 8G). The plotted data show that the relative fluorescence of intracellular OG-Lp rapidly decreases, whereas that of DiI-LDL remains constant during a 60 min chase. From these observations, we conclude that Lp exits the cells with a $t_{½}$ of ~13 min, which is in good agreement with that of Tf (Mayor et al., 1993; Ghosh et al., 1994). Similar to OG-Lp, the amount of intracellular OG-RAP decreased during the chase. However, ~50% of the initial amount of RAP was not resecreted (Fig. 8G). The clearance of intracellular Lp strongly suggests that most Lp is resecreted, whereas half of internalized amount of RAP exits the cell after passage through the ERC.

Taken together, all the results indicate that Lp uptake is specifically mediated by LpR. In addition to insect lipoprotein, LpR is capable of binding and internalizing human RAP. In contrast to LDL, which ends up in lysosomes, ligands that are internalized by LpR are not destined for lysosomal degradation. As a result of the

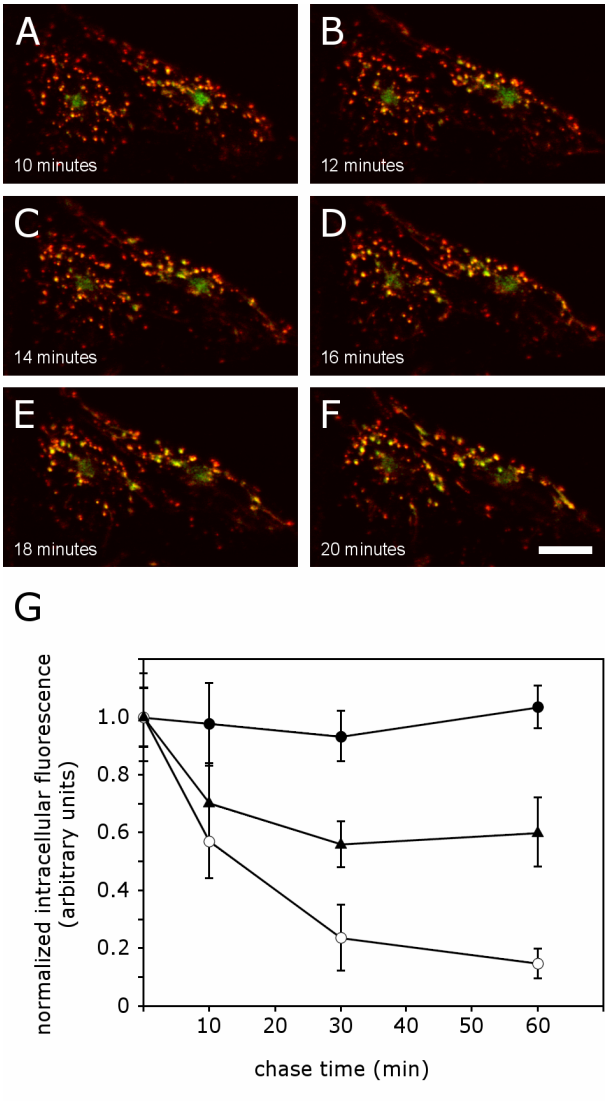

Figure 8. Lp exits CHO(LpR) cells with a $t_{½}$ of ~13 min; approximately 50% of internalized RAP is resecreted. Living CHO(LpR) cells, preincubated with DiI-LDL and OG-Lp, were observed in an aluminium chamber with confocal laser microscopy during a chase for 20 min. Digital photos were collected 10 min after initiating the chase at 2 min time intervals (A-F) to visualize the intracellular distribution of LDL (red) and Lp (green). To quantify the resecretion of internalized fluorescently-labeled lipoproteins, CHO(LpR) cells were fixated immediately after preincubated with DiI-LDL (filled circles), OG-RAP (filled triangles), or OG-Lp (open circles), and after a chase for 10, 30 or 60 min (G). Images were recorded using confocal laser microscopy and analyzed with Scion Image software. The data points are geometric means of the measured relative intensity of total cells. Of each time point, 10 to 17 images were taken with 4 to 33 cells per image, corresponding to more than 200 cells per data point. The mean values were normalized to the measurement immediately after the preincubation. The data were processed with Microsoft Excel and plotted with SigmaPlot. Values are means ± s.e.m. of 10 or more digital images (G). Bar, 20 μm (F).

intracellular pathway of the receptor, LpR-coupled ligands follow a transferrin-like intracellular recycling route.

Discussion

A generally accepted property of LDLR family members is their ability to endocytose ligands and transport them to sorting endosomes. Due to the low lumenal pH, the internalized ligands are released from their receptors and transported to lysosomes for degradation. The receptors are recycled back to the cell surface via the ERC and thereby

escape lysosomal hydrolysis. In vertebrates, LDLR-mediated endocytosis of LDL is essential for plasma cholesterol homeostasis. In consistence with the expected fate of lipoproteins, LDL is degraded in lysosomes and resulting lipid components are released into the cytoplasm (Brown and Goldstein, 1986). Here we report a novel intracellular distribution and fate of an apoB homologue-containing lipoprotein, Lp, which escapes its expected degradation in LpR-transfected CHO cells. Recycling of exchangeable apolipoproteins upon receptor-mediated endocytosis is not unique [e.g. apolipoprotein C (Heeren et al., 1999), and E (Fazio et al., 1999; Rensen et al., 2000)]; however, the recycling of non-exchangeable apolipoprotein, such as apoB, has not yet been described to occur in mammalian cells. On the basis of the results presented in this study, we conclude that, despite the non-exchangeable protein matrix being the sole apolipoprotein compound of Lp, the intracellular route of this lipoprotein deviates from the classic lysosome-directed pathway.

CHO cells that are transfected with LpR cDNA mediate endocytosis of Lp, however, the ligand remains in complex with the receptor in sorting endosomes. Several LDLR family member mutants have been constructed to identify the responsible domains and investigate the biochemical mechanisms involved in ligand uncoupling due to an acidic pH (Davis et al., 1987; Mikhailenko et al., 1999). Here we present evidence for the first naturally occurring LDLR family member, the ligands of which remain coupled to LpR in sorting endosomes and are consequently transported to the ERC to be eventually resecreted in a transferrin-like manner.

Resecretion of Lp after endocytosis is consistent with the role for Lp as a reusable shuttle for selective lipid delivery. The major difference between insect and mammalian lipoproteins is the selective mechanism by which insect lipoproteins transfer their hydrophobic cargo. Dependent on the physiological situation, circulating Lp particles serve as either diacylglycerol (DAG) acceptors at the insect fat body during adult stage-restricted flight activity, or donors during dietary lipid storage in the fat body of larval and young adult insects (for reviews see Van der Horst, 1990; Ryan and Van der Horst, 2000; Van der Horst et al., 2001, 2002). In the latter case, endocytic uptake of Lp seems to conflict with the selective unloading of lipids from Lp to fat body cells without concurrent degradation of the ligand (Arrese et al., 2001). In experiments in which fat body tissue from young adult locusts was incubated with Lp containing ^{3}H-labeled DAG and apolipoproteins, ^{3}H-DAG appeared to be taken up selectively without substantial concomitant accumulation of the radiolabeled apolipoproteins (Dantuma et al., 1997). Endocytosis of Lp for lipid storage in fat body cells had earlier been postulated for the insect *Ashna cyanea* (Bauerfeind and Komnick, 1992). However, thus far, evidence for recycling of the ligand had not been described. Our observations with fluorescently-labeled Lp strongly support that, despite receptor-mediated internalization of the ligand, Lp can be used as a reusable shuttle in both physiological conditions. Moreover, we provide preliminary evidence for the existence of a novel selective lipid-uptake mechanism mediated by an LDLR homologue that takes place intracellularly.

Despite structural homology between LDL and Lp at the protein level, we have shown that LpR specifically internalizes the insect lipoprotein, whereas LDLR exclusively mediates uptake of LDL. In addition to Lp, LpR shows a relatively high affinity for human RAP; a feature that is not shared by LDLR (Medh et al., 1995). However, all other members of the LDLR family have been observed to bind RAP with high affinity and internalize this ligand (Neels et al., 1998). The ability of LpR to bind

human RAP is in line with the presence of a RAP homologous gene identified in the *Drosophila* genome (Adams et al., 2000).

Transition of internalized Lp to the ERC is mediated by the membrane-spanning LpR in analogy to Tf recycling (Yamashiro et al., 1984). In contrast to the uncoupling of mammalian LDL from LDLR in sorting endosomes, Lp remains attached to its receptor despite the decrease in luminal pH. Endosome tubulation followed by iterative fractionation of membrane-anchored recycling receptors results in efficient receptor recycling by default (Dunn et al., 1989; Verges et al., 1999). Consequently, ligands that remain coupled to such receptors are recycled as well. Davis et al. (1987) showed that the EGF-precursor homology domain of LDLR is responsible for acid-dependent ligand dissociation. In addition, Mikhailenko et al. (1999) produced a VLDLR mutant, the EGF-precursor homology domain of which was deleted. They demonstrated that, in contrast to wild-type VLDLR, RAP did not dissociate from the mutant receptor after internalization and was not degraded. By using RAP as well as Lp, we show that LpR is capable of transporting physiologically unrelated ligands to the ERC, despite having a typical ligand-dissociating EGF-precursor homology domain. Our results combined with earlier observations using ^{3}H-labeled Lp to incubate fat body cells indicate that LpR-mediated recycling of Lp plays a physiologically relevant role in lipid storage (Dantuma et al., 1997). A selective lipid extraction mechanism would significantly reduce degradation as well as energy-consuming synthesis of reusable Lp.

Cellular uptake of Lp and human RAP by LpR results in an intracellular distribution of both ligands that deviates from the classic lysosomal delivery of mammalian lipoproteins in CHO cells. These observations propose a novel mechanism for ligand-uptake by an LDLR family member that is present in insects. It has been suggested that specific mammalian tissues may selectively take up lipoprotein-bound components with LDLR homologous receptors (e.g. LRP), however, without endocytosis of the ligand (Vassiliou et al., 2001; Swarnakar et al., 2001). Additionally, alternative functions for LDLR that deviate from the classic lysosomal lipoprotein delivery could also depend on the developmental stage or type of tissue (Dehouck et al., 1997). Our model system using LpR and CHO cells provides a powerful tool to study the molecular basis for the intracellular distribution and fate of ligands that are internalized by LDL receptors, as well as the function of individual receptor domains. An important issue to be solved remains the understanding of the molecular basis for the difference in targeting behavior of the mammalian and insect receptors. Although LDLR and LpR share a 57% sequence similarity, small differences in receptor domains might determine the fate of bound ligands. Whereas the ligand-binding domain of LDLR comprises seven cyteine-rich repeats, LpR has eight of these modules. The larger ligand-binding domain could cause a more stable ligand-receptor interaction, preventing acid-induced uncoupling in the endosomal compartment that is mediated by the EGF-precursor homology domain. In addition, the twelve C-terminal amino acids of the cytoplasmic tail of LDLR are completely different compared to those of LpR. Moreover, the intracellular portion of LpR has an additional ten amino acids. These residues could possibly interact with cytosolic components involved in processes that direct ligand or receptor distribution. Further analysis of insect lipoproteins and receptors, as well as the construction of hybrid receptors that are composed of (parts of) insect and mammalian receptors, will provide new insights into the understanding of molecular mechanisms that regulate lipoprotein binding and lipid uptake in mammals.

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CHAPTER 3

III

Lipophorin receptor-mediated lipoprotein endocytosis in insect fat body cells

Dennis Van Hoof, Kees W. Rodenburg and Dick J. Van der Horst

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CHAPTER 3

Lipophorin receptor-mediated lipoprotein endocytosis in insect fat body cells

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Abstract

Lipophorin (Lp) in the circulation of insects is able to selectively deliver lipids to target tissues in a non-endocytic manner. In *Locusta migratoria*, a member of the low-density lipoprotein (LDL) receptor (LDLR) family has been identified and shown to mediate endocytosis of Lp in mammalian cells transfected with the cDNA of this receptor. This insect lipophorin receptor (LpR) is temporally expressed in fat body tissue of young adult as well as larval locusts as shown by Western blot analysis. Fluorescence microscopy revealed that fat body cells internalize fluorescently-labeled Lp and human receptor-associated protein (RAP) only when LpR is expressed. Expression of LpR is down-regulated on the fourth day after an ecdysis. Consequently, Lp is no longer internalized. By starving adult locusts immediately after ecdysis, we were able to prolong LpR expression. In addition, expression of the receptor was induced by starving adults after down-regulation of LpR. These results suggest that LpR mediates endocytosis of Lp in fat body cells, and that expression of LpR is regulated by the demand of fat body tissue for lipids.

Introduction

Whereas mammals rely on a wide array of lipoproteins with different compositions and functions (Frayn, 1996), insects make use of a single type of lipoprotein, lipophorin (Lp; for review see Van der Horst et al., 2002), to effect the transport of lipids through the circulation. Lp comprises diacylglycerol and phospholipids as major lipid classes. The protein matrix consists of two non-exchangeable apolipoproteins, apolipophorin (apoLp)-I and apoLp-II, that are derived from a common precursor protein through post-translational cleavage (Weers et al., 1993; Bogerd et al., 2000). Sequence and domain structure analysis indicate that this precursor protein is homologous to mammalian apolipoprotein B-100 (apoB-100), the non-exchangeable protein component of very-low-density lipoprotein (VLDL) and its resulting low-density lipoprotein (LDL), and that both proteins have emerged from an ancestral gene (Babin et al., 1999; Mann et al., 1999; Segrest et al., 2001).

Lp is secreted by the insect fat body, an organ combining many of the functions of mammalian liver and adipose tissue (Locke, 1998). Similar to mammalian adipose tissue, the fat body retains large intracellular lipid depots that provide the fuel for energy-demanding tissues. Circulatory Lp is able to take up lipids released from the fat body cells and to selectively unload its lipid cargo at target tissues without endocytosis

and lysosomal degradation, and thus functions as a re-useable shuttle (for reviews see Van der Horst et al., 1993; Soulages et al., 1994; Ryan and Van der Horst, 2000; Van der Horst et al., 2002).

In spite of the concept of selective lipid transfer mediated by Lp, a novel member of the LDL receptor (LDLR) family was identified in *Locusta migratoria* (Dantuma et al., 1999). The domain structure composition of this insect lipophorin receptor (LpR) is identical to that of the mammalian VLDL receptor (VLDLR) and both have eight consecutive ligand-binding repeats. A similar receptor was found in the mosquito *Aedes aegypti* (Cheon et al., 2001). In a stably-transfected CHO cell line, locust LpR was shown to bind and internalize specifically Lp, but not human LDL (Van Hoof et al., 2002). In contrast to the lysosomal fate of ligands internalized by mammalian lipoprotein receptors, endocytosed Lp was observed to escape from degradation after LpR-mediated endocytosis. Both the occurrence of LpR in the insect (Dantuma et al., 1999; Cheon et al., 2001) and its functioning in a mammalian cell line (Van Hoof et al., 2002) suggested that internalization of Lp via receptor-mediated endocytosis may be a physiologically relevant process (Dantuma et al., 1997).

Using fluorescence microscopy, in this study we demonstrate that fat body tissue of young adult and larval locusts is able to internalize fluorescently-labeled Lp via LpR. In addition, similar to mammalian VLDLR, this receptor appears capable of internalizing human receptor-associated protein (RAP). On the fourth day after the energy-consuming process of ecdysis, expression of LpR drops below detectable levels in young adult as well as larval locusts. Fat body tissue excised from these insects has lost the ability to endocytose Lp. Down-regulation of LpR was postponed when adults were starved immediately after ecdysis. In addition, starving adult locusts after down-regulation of LpR induced expression of the receptor. Taken together, these results suggest that LpR mediates endocytosis of Lp in insect cells, and provide evidence for regulation of LpR expression under specific physiological conditions. The endocytic property of LpR is compared to that of VLDLR, and its proposed lipoprotein recycling function observed in mammalian cells as well as its possible role in lipid storage in insects are discussed.

Materials and methods

Antibodies, reagents and proteins

Precision protein standards prestained broad range marker (Bio-Rad), alkaline phosphatase-conjugated affinipure goat-anti-rabbit IgG, DiI(C_{18}(3)) (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine percholate) and Oregon Green 488 (OG) carboxylic acid (Molecular Probes), DAPI (Roche Diagnostics), leupeptin, aprotinin and BSA (Sigma), ^{125}I[iodine] (3.9 GBq/ml; Amersham Pharmacia Biochem), Oil Red O (Chroma), Coomassie Brilliant Blue (Serva), trypsin-EDTA (Invitrogen), and chloramine-T (Merck) were obtained from commercial sources. Lp was isolated from locust hemolymph by ultracentrifugation (Van Hoof et al., 2002). Membrane proteins of wild-type (wt) CHO and LpR-transfected CHO (CHO(LpR)) cells were isolated as described by Van Hoof et al. (Van Hoof et al., 2002). Polyclonal rabbit-anti-LpR 9218 antibody was raised against a synthetic peptide representing the unique C-terminal 19 amino acids (865-883) of LpR (Van Hoof et al., 2002), and polyclonal rabbit-anti-

LpR 2189/90 antibody was raised against a synthetic peptide representing the unique very N-terminal 20 amino acids (34-53) of the first cysteine-rich repeat of LpR. Human RAP was a generous gift from Dr. Michael Etzerodt (IMSB, Aarhus University, Aarhus, Denmark).

Insects

Insects were reared under crowded conditions in a temperature-controlled environment at 30°C with a relative humidity of 40% and a 12 h light/dark cycle. Immediately after ecdysis, male and female fifth instar (L_5) larvae were transferred to separate cages to obtain synchronized larval fat body. The same procedure was used to obtain synchronized adult male and female locust fat body, after the imaginal ecdysis. When starved, individual animals were transferred to separate cages and given access to water to prevent dehydration.

In vitro incubation of fat body tissue with fluorescently-labeled ligands

Lp (1 mg/ml) was fluorescently labeled in PBS with 50 µl/ml DiI in DMSO (3 µg/µl) at 37°C under continuous stirring for 2.5 h. Lp and RAP (1 mg/ml) were labeled with 20 µl/ml OG dissolved in DMSO (1 µg/µl) at room temperature under continuous stirring for 1 h according to the manufacturer's instructions. DiI and OG-labeled Lp (DiI-Lp and OG-Lp, respectively) were purified with Sephadex G-25 PD-10 columns (Amersham Pharmacia Biotech) to separate fluorescently-labeled ligand from free fluorescent label and replace the PBS by incubation medium (10 mM HEPES, 50 mM NaCl, 10 mM KCl, 5 mM $CaCl_2$, 2 mM $MgSO_4$, pH 7.4). OG-labeled RAP (OG-RAP) was dialyzed against incubation medium using standard cellulose membrane (Medicell International). Fat body tissue was incubated with 10 µg/ml DiI-Lp, 25 µg/ml OG-Lp, or 3.6 µg/ml OG-RAP for 30 min at 32°C for endocytic uptake. Tissue was rinsed in incubation medium and immediately fixated in 4% paraformaldehyde diluted in PBS for 30 min at room temperature. Where indicated, prior to fixation, fat body tissue was incubated with 0.05% trypsin in 0.35 mM EDTA for 5 min at room temperature, and washed thoroughly in incubation medium. For cell surface binding, fat body tissue was incubated with fluorescently-labeled Lp for 1 h at 4°C, thoroughly washed in incubation medium and fixated as described. For endocytosis of surface-bound Lp, fat body tissue was preincubated with OG-Lp for 30 min at 4°C, thoroughly washed, and then incubated in medium without fluorescently-labeled Lp for 30 min at 32°C, followed by fixation as described. After fixation, fat body tissue was incubated with 0.25 µg DAPI per ml PBS for 30 min at room temperature to stain the nuclei of the cells.

Microscopy and image processing

Cover slips with fixated tissue were mounted in mowiol and examined on a light and fluorescence Axioscop microscope (Zeiss) with a Hg HBO-50 lamp and a Plan-Neofluar 100×/1.30 oil lens. Using UV and FITC/TRITC filters, digital images were recorded with a DXM 1200 digital camera and ACT-1 version 2.00 software (Nikon). Images of centrally-localized nuclei and peripherally-distributed endocytic vesicles of the same area were obtained sequentially at their respective confocal planes. Corresponding images of nuclei and vesicles were subsequently processed and merged using PaintShop pro 7.00 (Jasc Software).

Western blot analysis of fat body membrane protein extracts

Fat body tissue from male and female larvae and adult locusts was excised in incubation medium containing protease inhibitors. Fat body tissue of 3 to 5 individuals were pooled and fractionated by thoroughly resuspending and vortexing, and kept on ice during the following purification steps. Samples were centrifuged for 10 min at 10°C at 15000 *g* to separate the fractionated cells from the released lipids. The fat cake was removed with a toothpick and the supernatant was discarded after which the pellet was resuspended in protease inhibitor-containing incubation medium. The samples were centrifuged again at 15000 *g* for 10 min at 10°C after which the supernatant was removed and the remaining lipids were discarded with a tissue. The lipid-depleted pellets were resuspended in 40 to 80 µl CHAPS buffer (20 mM HEPES, 124 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 2.5 mM Na_2HPO_4, 1.2 mM $MgSO_4$, 1 mM EDTA, 0.1 mM benzamidine, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 1% CHAPS), to resuspend the pellet. The suspension was incubated for 10 min on ice, and spun down at 15000 *g* for 10 min at 10°C. Supernatant containing 5.0 µg of total membrane protein was transferred to a clean Eppendorf tube and either heated for 5 min at 95°C in Laemmli buffer (Laemmli, 1970) or directly dissolved in modified Laemmli buffer (containing 0.025% SDS and no disulfide bond-reducing reagents) prior to separation by SDS-PAGE in a 10% polyacrylamide gel. The separated membrane proteins were transferred to polyvinylidene fluoride membrane (Millipore) and incubated with rabbit-anti-LpR 9218 (1:2000) or 2189/90 (1:100) antibody for 2 h, followed by 1 h alkaline phosphatase-coupled goat-anti-rabbit incubation. Bound second antibody was visualized by incubating the blot in TSM buffer, containing 100 mM Tris-HCl, 100 mM NaCl, 10 mM $MgAc_2$, 50 µg/ml p-nitro blue tetrazolium chloride (NBT; Boehringer Mannheim), 25 µg/ml 5-bromo-4-chloro-3-indoyl-phosphate p-toluidine (BCIP; Roche Diagnostics), pH 9.0.

Ligand blot with ^{125}I-RAP

^{125}I-RAP was prepared using chloramine-T according to Rodenburg et al. (Rodenburg et al., 1998), resulting in a specific labeling activity of ~45000 cpm/ng protein. Polyvinylidene fluoride membrane, containing 20.0 µg per lane of total membrane proteins that were separated by SDS-PAGE under non-reducing conditions, was incubated over night with 12 nM ^{125}I-RAP in binding buffer (20 mM HEPES, pH 7.4, 50 mM NaCl, 2.5 mM $CaCl_2$, 0.5% (w/v) BSA) after which the blot was washed several times with binding buffer. RAP binding was detected using a PhosphorImager (Molecular Dynamics), and visualized using MD ImageQuant software version 3.3 (Molecular Dynamics). RAP-receptor binding was quantified by determining the radioactivity in those parts of the ligand blots that corresponded to receptor-bound complexes, using the data from the PhosphorImager.

Results

In vitro endocytosis of Lp by fat body tissue of young adult locusts

In contrast to the mechanism by which Lp selectively unloads lipids at target tissues, endocytosis of Lp may provide an alternative mechanism for uptake of lipid

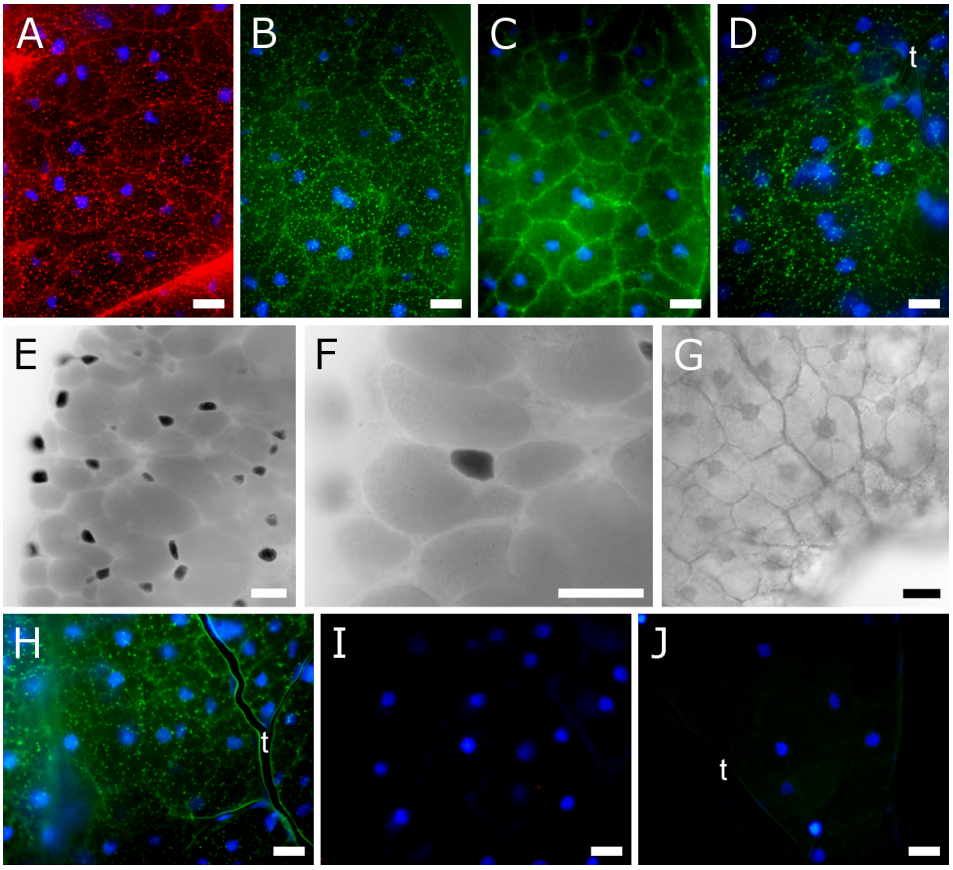

Figure 1. Multi-color fluorescence microscopic images of locust fat body tissue obtained with FITC and TRITC filters combined with a UV filter. On the first day after imaginal ecdysis, fat body tissue was excised from male locusts and incubated with DiI-Lp (A) or OG-Lp (B-D) for 30 min at 32°C. Two FITC images were captured of the same area of fat body tissue peripherally below the cell surface (B) and at the confocal plane of the nuclei (C). Prior to fixation, fat body tissue was incubated with trypsin and washed thoroughly (D). Light microscopic images were merged with fluorescence microscopic images of fat body tissue excised from adult males, immediately after imaginal ecdysis. (E) After fixation, the lipid droplets were stained with Oil Red O (grey) and visualized with light microscopy. Nuclei were stained with DAPI (black) and visualized with fluorescence microscopy using a UV filter. The colors of the DAPI fluorescence image were inverted and reduced to a grey scale before merging the light and fluorescence images. (F) Detailed image of a single nucleus derived from panel (E). Total cell proteins were stained with Coomassie Brilliant Blue and visualized with light microscopy (G). Images of fat body tissue excised from females on the first day (H) or males on the fourth day after imaginal ecdysis (I and J) that was incubated for 30 min at 32°C with OG-Lp (H and J) or DiI-Lp (I). DiI-Lp is shown in red (A and I); OG-Lp is shown in green (B-D, H, and J); nuclei stained with DAPI are shown in blue (A-D, and H-J), black (E and F), or grey (G); tracheae are indicated with **t** (D, H, and J). All TRITC and FITC images were overlaid and merged with images of the nuclei that were stained with DAPI and visualized with a UV filter. Bars, 20 µm.

components in fat body cells. Therefore, Lp was fluorescently labeled with DiI (DiI-Lp) to visualize the lipoprotein after incubation of fat body tissue that was excised from young adult male locusts within 24 h after ecdysis. Incubation of fat body tissue with incubation medium containing DiI-Lp resulted in a punctate staining pattern characteristic for endocytosis (Fig. 1A). To investigate whether, in addition to the lipid,

also the apolipoprotein component was internalized, the amine-reactive probe Oregon Green 488 (OG) was used to label apoLp-I and apoLp-II. Analogous incubation conditions with OG-labeled Lp (OG-Lp) resulted in a similar endocytic uptake pattern (Fig. 1B). Scanning vertically through incubated fat body tissue revealed that Lp-containing vesicles are peripherally localized in the cells (Fig. 1C). Treatment of tissue with trypsin prior to fixation did not alter the punctate staining pattern, verifying that Lp is encapsulated in membranes (i.e. endocytic vesicles; Fig. 1D).

The trophocyte, or adipocyte, is the main cell type that constitutes the fat body, and is used for storage of lipids and glycogen (Locke, 1998). The size and shape of trophocytes are predominantly determined by the lipid droplets that fill up almost the entire intracellular space (Fig. 1E and F). As a result, cytoplasm is mainly situated peripherally below the cell surface and between the lipid droplets, whereas the nuclei are predominantly located in the cell center (Fig. 1G). Incubation of young adult female fat body tissue with DiI-Lp (data not shown) or OG-Lp (Fig. 1H) resulted in identical staining patterns as observed with male fat body tissue, which suggests an uptake mechanism that is present in both sexes. In contrast, fat body tissue excised from adults on the fourth day or later after ecdysis remained devoid of fluorescently-stained endocytic vesicles when incubated with DiI-Lp or OG-Lp (Fig. 1I and J, respectively). These findings suggest a down-regulation of the ability to internalize Lp via endocytosis that occurs on the fourth day after imaginal ecdysis.

Endocytosis of Lp by fat body cells is mediated by LpR

To investigate the involvement of a receptor in Lp endocytosis, fat body tissue was incubated at 4°C at which receptor-mediated endocytosis is inhibited, whereas cell surface binding still occurs (Dunn et al., 1980). As shown in Fig. 2A and B, the internalization of both DiI-Lp and OG-Lp was prevented. Transfer of tissue that was

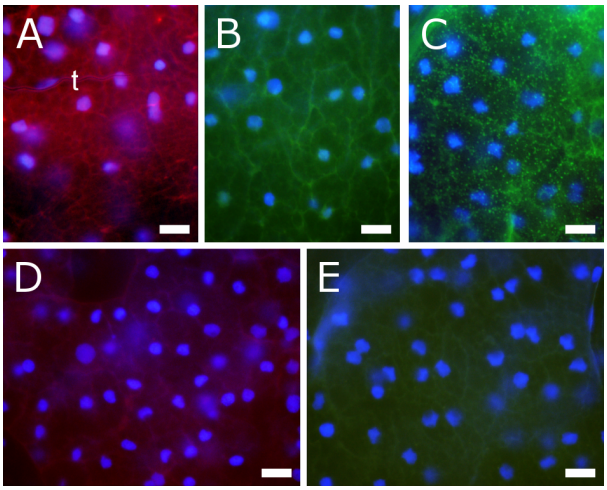

Figure 2. Fat body tissue was excised from adult male locusts on the first day after ecdysis and incubated for 1 h at 4°C with DiI-Lp (A) or OG-Lp (B) in incubation medium. After preincubation with OG-Lp at 4°C, fat body tissue was transferred to medium without fluorescently-labeled Lp and incubated at 32°C (C). Prior to incubation with fluorescently-labeled Lp, fat body tissue excised from males on the first day after imaginal ecdysis was preincubated for 30 min at 32°C with 2.5 mg/ml unlabeled Lp. Subsequently, the preincubation medium was replaced by incubation medium containing the same concentration of unlabeled Lp supplemented with either 10 µg/ml DiI-Lp (D) or 25 µg/ml OG-Lp (E) and incubated for an additional 30 min at 32°C. Digital images were taken from the confocal plane peripherally below the cell surface with TRITC or FITC filters for DiI-Lp and OG-Lp, respectively. DiI-Lp is shown in red (A and D); OG-Lp is shown in green (B, C, and E); nuclei stained with DAPI are shown in blue (A-E); tracheae are indicated with **t** (A). All TRITC and FITC images were overlaid and merged with images of the nuclei that were stained with DAPI and visualized with a UV filter. Bars, 20 µm.

preincubated with OG-Lp at 4°C to medium without Lp resulted in the formation of OG-Lp-containing endocytic vesicles when the temperature was raised to 32°C (Fig. 2C). At 32°C, a hundred-fold excess of unlabeled Lp prevented endocytic uptake of DiI- and OG-labeled Lp (Fig. 2D and E, respectively). These observations imply that endocytosis of Lp by fat body cells of young adult locusts is mediated by a receptor.

LpR has recently been shown to mediate endocytic uptake of Lp in CHO cells that were stably transfected with an expression vector harboring LpR cDNA (Van Hoof et al., 2002). Consequently, LpR was supposed to mediate endocytosis of Lp in these young adult locusts. The presence of LpR was analyzed using cell membrane extracts from adult locusts at defined time points after ecdysis. Membrane proteins were separated by SDS-PAGE under reducing conditions, and immunodetected with anti-LpR 9218 antibody raised against the cytoplasmic tail of LpR that is unique for insect lipophorin receptors (Van Hoof et al., 2002). Under reducing conditions, LpR has a

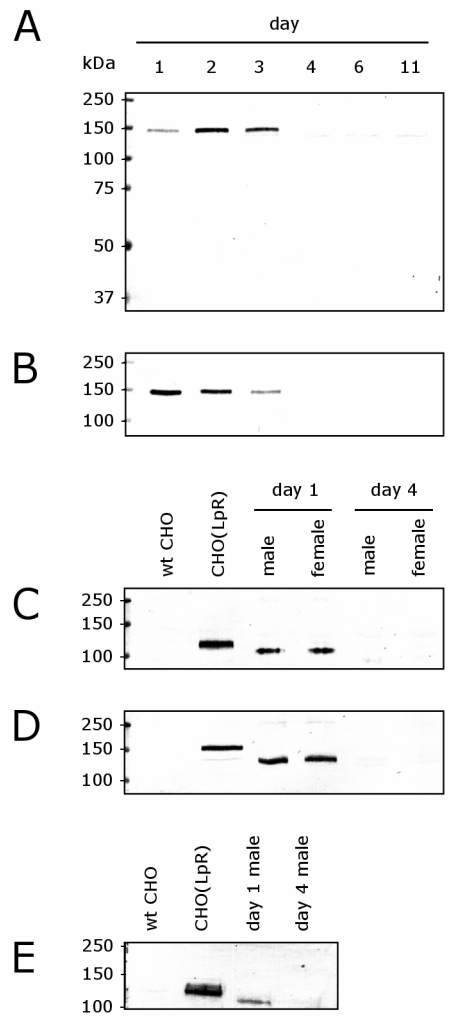

Figure 3. Fat body tissue was excised from male (A) and female (B) locusts after imaginal ecdysis on the days indicated and the membrane proteins separated by SDS-PAGE. (C-E) Membrane proteins were obtained from wt (lane 1) and CHO(LpR) (lane 2) cells harvested from culture flasks, and fat body of day one (C and D, lane 3 and 4; E, lane 3) or day four (C and D, lane 5 and 6; E, lane 4; controls) adult locusts. Samples were either dissolved in modified Laemmli buffer (Laemmli, 1970) and directly subjected to SDS-PAGE under non-reducing conditions (C and E), or heated for 5 min at 95°C in Laemmli buffer (D) and then separated by SDS-PAGE. LpR was detected by immunoblotting using anti-LpR 9218 antibody (A-D) or anti-LpR 2189/90 antibody (E). The molecular weight markers (kDa) are indicated on the left of each panel.

molecular weight of approximately 140 kDa and is expressed in both males and females (Fig. 3A and B, respectively). In addition, the blots show that LpR is expressed during the first three days after imaginal ecdysis, which is in agreement with the capability of young adult fat body tissue to endocytose Lp (Fig. 1A and B). On the fourth day, expression of LpR drops below detectable levels (Fig. 3A and B), which coincides with the absence of fluorescently-labeled Lp-containing endocytic vesicles in fat body tissue of these animals (Fig. 1I and J).

Non-reduced LpR obtained from fat body cells (Fig. 3C, lane 3 and 4) has a higher electrophoretic mobility in SDS-PAGE compared to reduced LpR (Fig. 3D, lane 3 and 4), indicating the presence of multiple disulfide bonds. The molecular weight of reduced LpR is higher than the theoretical 98 kDa based on the amino acid sequence (Dantuma et al., 1999), suggesting that endogenous *L. migratoria* LpR is glycosylated, like all other LDLR family members (Russell et al., 1984; Neels et al., 1998). LpR isolated from CHO(LpR) cells (Fig. 3C and D, lane 2) has a higher molecular weight compared to that obtained from fat body cells (Fig 3C and D, lane 3 and 4), which is most likely the result of a different degree of glycosylation between the two different cell types (Marz et al., 1995; Altmann et al., 1999). Immunodetection with anti-LpR 2189/90 antibody raised against the 20 N-terminal amino acids of the first cysteine-rich repeat of LpR gave a similar result with non-reduced membrane extracts of CHO(LpR) and fat body cells as shown in Fig. 3C (Fig. 3E). These data additionally support that, despite a difference in molecular weight, the recognized membrane proteins from both cell types are LpR.

RAP is a ligand for LpR

RAP serves as a chaperone to assist the folding of LDLR family members and prevents premature binding of ligands in the endoplasmic reticulum (Bu et al., 2000). It has been shown to inhibit binding of ligands to lipoprotein receptors (Herz et al., 1991; Kounnas et al., 1992; Battey et al., 1994), including LpR (Van Hoof et al., 2002). Fat body tissue of young adult locusts that was incubated with DiI-Lp or OG-Lp remained devoid of Lp-containing vesicles when a hundred-fold molar excess ratio of human RAP was added to the incubation medium (Fig. 4A and B, respectively). Inhibition of Lp endocytosis by RAP suggests that the protein serves as a ligand for LpR, and thus can also be internalized by fat body cells. Incubation of young adult fat body tissue with OG-labeled RAP (OG-RAP) resulted in a particulate pattern identical to that of endocytosed OG-Lp (compare Figs. 4C and 1B). Endocytosis of OG-RAP was completely inhibited with a hundred-fold molar excess ratio of either unlabeled Lp (Fig. 4D) or unlabeled RAP (Fig. 4E), suggesting that Lp and RAP bind to the same fat body receptor.

To confirm that the RAP-binding receptor is LpR, fat body cell membrane proteins separated under non-reducing conditions were transferred to polyvinylidene fluoride membrane and incubated with ^{125}I-labeled RAP. Immediately after adult ecdysis, fat body membrane extracts contain a single binding protein with a molecular weight of approximately 110 kDa (Fig. 4F, lane 1 and 2), which is identical to that of LpR under non-reducing conditions. These results strongly suggest that LpR is the only endocytic lipoprotein receptor expressed in this stage. In day four locust fat body tissue the RAP-binding protein is no longer significantly present (Fig. 4F, lane 3 and 4). In agreement with this finding, OG-RAP was not endocytosed by fat body tissue derived from these

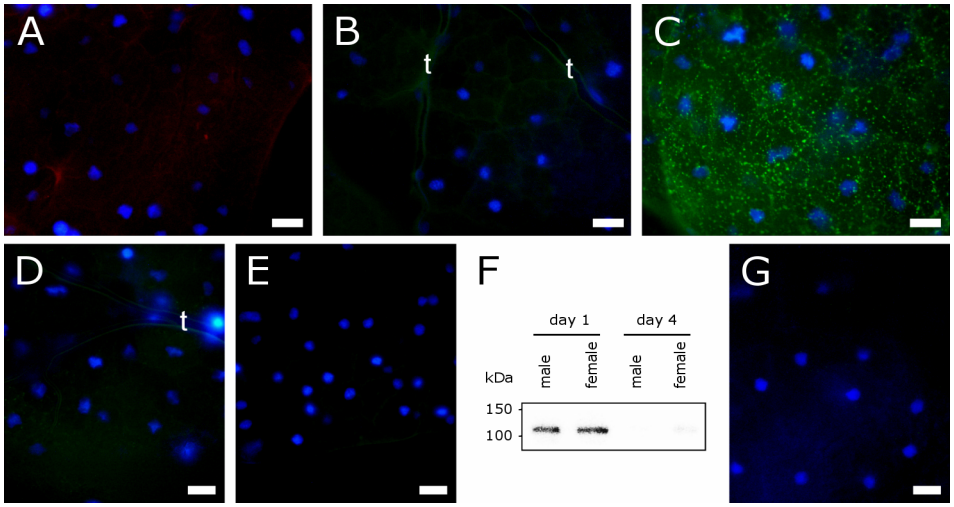

Figure 4. Fat body tissue, excised from locusts on the first day after imaginal ecdysis, was preincubated for 30 min at 32°C with incubation medium containing 0.36 mg/ml human RAP. After preincubation, the medium was removed and the tissue was subsequently incubated with DiI-Lp (A) or OG-Lp (B) in the presence of 0.36 mg/ml unlabeled human RAP. (C) OG-RAP was used to incubate fat body tissue of young adult locusts immediately after ecdysis. Prior to OG-RAP incubation, fat body tissue of the same developmental stage was preincubated with incubation medium containing either 2.5 mg/ml Lp (D) or 0.36 mg/ml human RAP (E). After removing the preincubation medium, the tissue was incubated with OG-RAP in the presence of a 100-fold molar excess ratio of unlabeled Lp (D) or RAP (E). (F) Fat body tissue was excised from locusts after imaginal ecdysis on the indicated days and membrane proteins were dissolved in Laemmli buffer containing 0.025% SDS and no reducing agents. The non-reduced proteins were subjected to SDS-PAGE, blotted onto polyvinylidene fluoride membrane and incubated with ^{125}I-labeled RAP after which the ligand blot was analyzed with a PhosphorImager. The molecular weight markers (kDa) are indicated on the left of the panel. (G) Fat body tissue of day four adult male locusts was incubated with 3.6 µg/ml OG-RAP for 30 min at 32°C (G). DiI-Lp is shown in red (A); OG-Lp is shown in green (B); OG-RAP is shown in green (C-E, and G); nuclei stained with DAPI are shown in blue (A-E, and G); tracheae are indicated with **t** (B and D). All TRITC and FITC images were overlaid and merged with images of the nuclei that were stained with DAPI and visualized with a UV filter. Bars, 20 µm.

adult locusts (Fig. 4G). Taken together, these findings confirm that, in addition to Lp, fat body cells are able to internalize RAP via LpR-mediated endocytosis.

LpR mediates Lp endocytosis in fat body cells of larvae

The finding that LpR is expressed after the imaginal ecdysis raises the question whether a similar up and down-regulation of the receptor also occurs in earlier developmental stages. Similar to adults (Fig. 5A, lane 1 and 2), LpR is highly expressed in L_5 larvae immediately after ecdysis (Fig. 5A, lane 3 and 4), and is down-regulated on the fourth day (Fig. 5A, lane 5 and 6). Expression of LpR in L_5 larvae implies that the fat body tissue is capable of internalizing Lp. On the first day after ecdysis to L_5, larval fat body tissue was incubated with OG-Lp resulting in a similar particulate staining pattern (Fig. 5B) as observed for young adults (Fig. 1B). On the fourth day, L_5 larvae have lost the ability to internalize OG-Lp under similar conditions (Fig. 5C), like day four adults (Fig. 1J), which coincides with the expression pattern of LpR (Fig. 5A). Collectively, these data on adults and larvae suggest that Lp is internalized by fat body cells during the first few days after each ecdysis, and that this endocytic uptake is mediated by LpR.

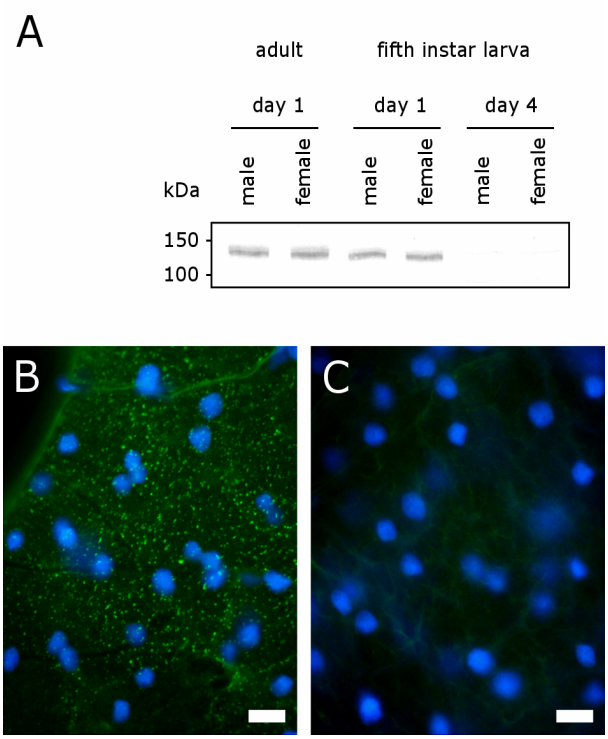

Figure 5. (A) Fat body tissue was excised from adult locusts and L_5 larvae on the days indicated. Membrane proteins were separated by SDS-PAGE under reducing conditions after which LpR was detected by immunoblotting using anti-LpR 9218 antibody. The molecular weight markers (kDa) are indicated on the left of the panel. Fat body tissue was excised from L_5 larvae on the first (B) and the fourth day (C) after ecdysis, and incubated with OG-Lp for 30 min at 32°C. OG-Lp is shown in green (B and C); nuclei stained with DAPI are shown in blue (B and C). All FITC images were overlaid and merged with images of the nuclei that were stained with DAPI and visualized with a UV filter. Bars, 20 µm.

LpR expression is induced by the demand of fat body tissue for lipids

In the first few days after each ecdysis, the fat body rapidly increases in volume due to the storage of a high amount of lipids derived from dietary intake. Combined with the expression pattern of LpR in young adult (Fig. 3A and B) and larval (Fig. 5A) locusts, this observation suggests that LpR mediates the rapid uptake of lipids after the depots in fat body cells have been depleted. Therefore, it was hypothesized that LpR expression may be regulated by a requirement of fat body cells for lipids. To support this hypothesis, adult locusts were starved to create a physiological condition in which the demand for lipids is increased.

First, we investigated whether the expression of LpR after ecdysis could be prolonged. Young adult locusts were starved immediately after imaginal ecdysis and membrane proteins were isolated on the fifth day, the developmental stage at which LpR expression in fed animals is below detectable levels (Fig. 3A and B). Western blot analysis with a high amount of membrane protein showed that, in contrast to fed animals (Fig. 6A, lane 3 and 4), LpR expression is retained in starved locusts (Fig. 6A, lane 5 and 6), suggesting that if lipid intake is postponed, the down-regulation of the receptor is delayed. In addition, the exoskeleton of starved locusts (Fig. 6B and C) remained less pigmented than that of fed locusts (Fig. 6D and E), indicating that components for pigmentation (e.g. carotenoids) are derived from dietary intake. With the high amount of membrane protein used for these Western blots, anti-LpR 9218 antibody also recognized a membrane protein of ~130 kDa, the electrophoretic mobility

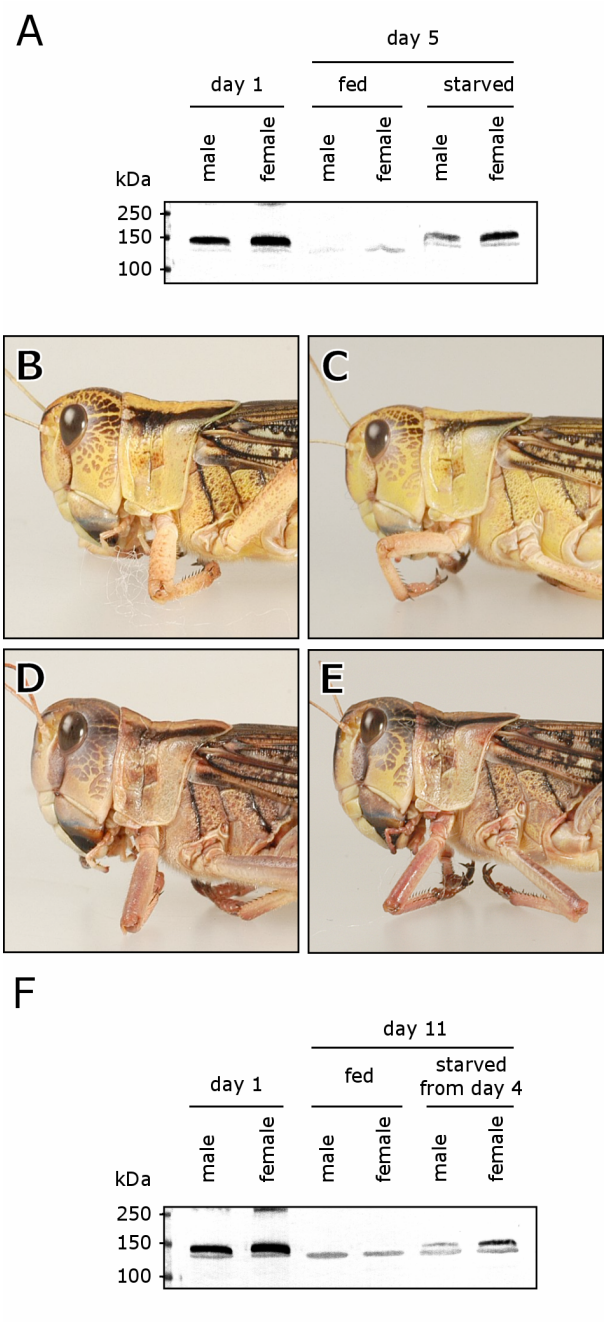

Figure 6. Twice the standard amount of fat body membrane proteins from male (lane 1, 3, and 5) and female (lane 2, 4, and 6) locusts were denatured in Laemmli buffer and subjected to SDS-PAGE after which LpR was detected by immunoblotting using anti-LpR 9218 antibody. (A) Fat body membrane proteins obtained from day one adult locusts (lane 1 and 2) and day five adult locusts that had either been normally fed (lane 3 and 4) or starved from the imaginal ecdysis until the day of excision (lane 5 and 6). Images of fed (B and C) and starved locusts (D and E) show differences in exoskeleton pigmentation. (F) Lane 1 and 2 contain fat body membrane proteins of day one adult locusts. Membrane proteins separated in lane 3 and 4 were obtained from adult locusts that had been normally fed for eleven days. Lane 5 and 6 contain membrane proteins from locusts that were fed for four days after imaginal ecdysis and then starved until the day of isolation. The molecular weight markers (kDa) are indicated on the left of each panel.

of which was not altered under non-reducing conditions (data not shown). This protein neither enabled adult fat body tissue of day four and later to visibly internalize Lp (Fig. 1I and J) or RAP (Fig. 4G), nor bound RAP after ligand blotting under non-reducing conditions (Fig. 4F), and therefore does not seem to function in Lp

endocytosis. However, anti-LpR 2189/90 antibody also recognized this protein (data not shown), suggesting that the protein is most likely structurally related to LpR.

Second, to obtain additional evidence for a regulation of LpR expression by lipid depletion, we investigated whether the expression could be induced after down-regulation had occurred. Adult locusts were starved from day four to eleven after ecdysis, whereafter fat body cell membrane proteins were isolated and analyzed as described above. Whereas LpR is absent in membrane extracts from fed locusts (Fig. 6F, lane 3 and 4), starved animals show a significant expression of LpR (Fig. 6F, lane 5 and 6). Although the amount of LpR per total amount of membrane protein appeared not as high as observed in animals immediately after ecdysis (Fig. 6A and B, lane 1 and 2) these data suggest that LpR expression can be induced by reducing the lipid stores in fat body cells.

Discussion

Lp has been proposed to selectively unload lipid cargo at the cell surface of target tissues (for reviews see Van der Horst et al., 1993; Soulages et al., 1994; Ryan and Van der Horst, 2000; Van der Horst et al., 2002), thus avoiding the lysosomal fate of endocytosed mammalian lipoproteins (for reviews see Goldstein et al., 1985; Mellman, 1996; Mukherjee et al., 1997; Hussain et al., 1999). Consequently, the process of lipid storage in fat body cells may be expected to proceed via selective lipid uptake. However, the expression of an insect LDLR family member at the surface of insect fat body cells implies that Lp can be additionally internalized via receptor-mediated endocytosis. This is also supported by the identification and characterization of an LpR that is expressed on the surface of mosquito oocytes and shown to bind Lp (Cheon et al., 2001).

Endocytosis of Lp has been demonstrated earlier for the dragonfly *Aeshna cyanea* (Bauerfeind and Komnick, 1992) and *L. migratoria* (Dantuma et al., 1997). The latter results, however, relate to internalization of Lp during a period in which LpR is not significantly expressed. The results of the present study provide evidence for an LpR-mediated endocytic uptake mechanism for Lp that is present during specific periods of development in the locust. Incubation experiments using excised fat body tissue and fluorescently-labeled Lp clearly show that, shortly after ecdysis, fat body cells are able to endocytose the complete particle; both the lipid moiety and protein matrix of Lp are internalized (Fig. 1A and B, respectively). The simultaneous expression of LpR (Fig. 3) strongly suggests that this receptor is responsible for Lp endocytosis. Like the intricate process of ecdysis, the appearance of LpR may be controlled by developmental hormones, resulting in a temporal up-regulation of LpR during critical periods in developmental stages in which endocytosis of Lp is needed. However, expression of LpR is also triggered by starvation (Fig. 6), which may be a response to rapidly replenish the depleted fat body reserves once lipids from dietary intake are available. Therefore, LpR expression is most likely not restricted to the first few days after an ecdysis, but may be induced after all lipid store-depleting processes (e.g. ecdysis, starvation and possibly sustained flight).

The simultaneous existence of the two distinct uptake mechanisms in fat body cells cannot be excluded. Whereas Lp is able to selectively unload its lipid cargo at the cell

surface of target tissues, receptor-mediated endocytosis of Lp may be a requisite to internalize components that are unable to diffuse through the cell membrane. In contrast to the selective lipid transfer mechanism, via which degradation of Lp is circumvented, endocytosis of the lipoprotein would imply that the complete particle is degraded in lysosomes, similar to mammalian LDL (for reviews see Goldstein et al., 1985; Mellman, 1996; Mukherjee et al., 1997; Hussain et al., 1999). However, experiments involving CHO(LpR) cells demonstrated that endocytosed Lp is resecreted without substantial degradation (Van Hoof et al., 2002). In fat body cells, preliminary pulse-chase experiments indicate that the particle may also be resecreted after endocytosis. Extensive incubation of excised tissue in insect growth medium following a pulse of fluorescently-labeled Lp resulted in a significant decrease in the number of Lp-containing vesicles (data not shown). However, these remaining vesicles were larger compared to the initial endosomes, which may be due to fusion and maturation of the endosomes into lysosomes (Stoorvogel et al., 1991; Dunn and Maxfield, 1992); thus degradation of Lp cannot be excluded. The fragility and typical composition of fat body tissue render it difficult to allow quantitation of Lp degradation or recycling. We are currently addressing this issue using LpR-transfected insect cell lines (e.g. Sf9), that provide a suitable alternative to determine the fate of endocytosed Lp in insect cells.

LpR and VLDLR share 58.2% amino acid sequence similarity and have an identical domain structure composition. The ligand-binding domains of both receptors comprise eight consecutive cysteine-rich repeats, and LpR (Fig. 4F; Van Hoof et al., 2002) as well as VLDLR (Battey et al., 1994; Simonsen et al., 1994) are capable of binding RAP. In spite of their apparent structural homology, LpR and VLDLR seem to function differently. Whereas LpR mediates endocytic uptake of lipoprotein in insect as well as mammalian cells, VLDLR is assumed to function extracellularly. Storage of VLDL-derived TAG is presumed to depend on the extracellular hydrolysis of TAG by lipoprotein lipase (LPL) after which the liberated free fatty acids are taken up by adjacent cells (Tacken et al., 2001). Such a mechanism would require neither internalization of the lipoprotein, nor the involvement of an endocytic receptor (i.e. VLDLR). On the other hand, LPL has been reported to bind to LDLR family members including VLDLR (Takahashi et al., 1992; Arraves et al., 1995). Therefore, it was suggested that LPL and VLDLR together promote retention of circulating VLDL via a process that takes place extracellularly (Tacken et al., 2001). However, the intracellular tail of VLDLR harbors the putative internalization signal sequence NPxY (Chen et al., 1990), like all other LDLR family members, including LpR. In addition, VLDLR was shown to function as an endocytic receptor for VLDL in CHO cells (Takahashi et al., 1992). Moreover, *in vivo* studies showed that ectopic expression of this receptor in mouse liver results in enhanced internalization of lipoproteins (Kobayashi et al., 1996; Van Dijk et al., 1998). Thus, VLDLR apparently has the ability to function as an endocytic receptor, like all LDLR family members. Possibly, VLDLR-mediated VLDL endocytosis provides a backup mechanism when extracellular hydrolysis of VLDL-derived TAG is prevented; for instance, LPL-deficient patients were reported to show neither abnormal energy metabolism, nor deprivation of subcutaneous fat (Eckel et al., 1989).

Earlier findings with LDLR and our observations with LpR show that alternative functions for lipoprotein receptors that deviate from the classic lysosomal delivery may depend on the type of cell or developmental stage at which the receptor is expressed (Van Hoof et al., 2002; Dehouck et al., 1997). Our results indicate that, in spite of the

ability of Lp to selectively unload lipids at target tissues, Lp can also be taken up by fat body cells via LpR similar to the mammalian LDL uptake system. In addition, evidence is provided for a physiological regulation of LpR expression that is dependent on the requirement of fat body tissue for lipids. Thus, LpR expression is most likely not restricted to the first days after ecdysis, but may also occur by virtue of a need of fat body cells to replenish their storage depots. Further research will elucidate whether fat body cells are able to resecrete Lp after LpR-mediated endocytosis. Transfection of insect cell lines (e.g. Sf9) with LpR cDNA will provide insight into the intracellular transport routes of lipoproteins and other ligands after endocytosis in insect cells. Perhaps more importantly, unravelling the means of lipid storage in the insect as a model may very well contribute to the understanding of lipid storage at the cellular level in general.

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CHAPTER 4

IV

Receptor-mediated endocytosis and intracellular trafficking of lipoproteins and transferrin in insect cells

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Submitted

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Receptor-mediated endocytosis and intracellular trafficking of lipoproteins and transferrin in insect cells

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Abstract

Since the characterization of the low-density lipoprotein (LDL) receptor, the intracellular pathways of ligands after receptor-mediated endocytosis have been studied extensively in mammalian cells. In insect cells, however, these pathways are largely unknown. We transfected *Drosophila* S2 cells with the human LDL receptor (LDLR) and transferrin receptor (TfR), which mediate endocytosis of LDL and transferrin, respectively. After endocytosis in mammalian cells, LDL is degraded in lysosomes, whereas transferrin is recycled. Fluorescence microscopy analysis revealed that LDL and Tf are internalized by S2 cells transfected with LDLR or TfR, respectively. In double transfectants simultaneously expressing LDLR and TfR, both ligands colocalize in endosomes immediately after endocytic uptake, as well as after a chase. These findings indicate that transferrin is not resecreted after endocytosis, and remains in intracellular vesicles in which LDL also resides. Similar to S2 transfectants, transferrin is retained in vesicular compartments after internalization by another insect model cell line (Sf9 cells). This suggests that insect cells do not possess the ability to recycle ligands that are resecreted in mammalian cells. The insect lipoprotein, lipophorin, is recycled after lipophorin receptor (LpR)-mediated endocytosis in mammalian cells. In contrast, when expressed in S2 cells, LpR does not elicit resecretion of internalized lipophorin, which implies that recycling is cell type specific. LpR is endogenously expressed by fat body tissue of *Locusta migratoria* immediately after an ecdysis. A chase following endocytosis of labeled lipophorin by isolated fat body tissue at this developmental stage resulted in a significant decrease of lipophorin-containing vesicles, indicative of recycling of the ligand.

Introduction

Mammalian cell lines have been extensively used to study receptor-mediated endocytosis and subsequent intracellular trafficking of various ligands. The distinct endocytic pathways of low-density lipoprotein (LDL) and di-ferric transferrin (Tf) are well characterized (for reviews see Mellman, 1996; Mukherjee et al., 1997; Maxfield and McGraw, 2004), and therefore commonly used as markers for numerous vesicular compartments in the cell. LDL and Tf bind to the LDL receptor (LDLR) and transferrin

receptor (TfR), respectively, that both locate in clathrin-coated pits. The ligand-receptor complexes enter the cell in vesicles that subsequently fuse with tubulo-vesicular sorting endosomes. Mild acidification of the vesicle lumen triggers the dissociation of LDL from its receptor (for reviews see Innerarity, 2002; Jeon and Blacklow, 2003), whereas Tf remains in complex with TfR and merely unloads its two iron-ions (for reviews see Mellman, 1996; Mukherjee et al., 1997; Maxfield and McGraw, 2004). In contrast to released LDL particles that are retained in the sorting endosome, most of the remaining membrane constituents (e.g. LDLR and TfR) enter the tubular extensions, that bud off to be delivered to the morphologically distinct endocytic recycling compartment (ERC) (Brown et al., 1982; Yamashiro et al., 1984; Mayor et al., 1993; Verges et al., 1999). As a result, Tf also accumulates in these large, perinuclear organelles, and eventually exits this compartments with a $t_{½}$ of ~7 min (Mayor et al., 1993; Ghosh et al., 1994). On the other hand, sorting endosomes mature into lysosomes in which LDL particles are completely degraded (Goldstein et al., 1985; Dunn et al., 1989; Stoorvogel et al., 1991).

LDLR is the prototype for a large class of endocytic transmembrane receptors expressed in mammals (Brown et al., 1997; Hussain et al., 1999). LDLR family members have also been identified and sequenced in the insects *Locusta migratoria* (Dantuma et al., 1999, Van Hoof et al., 2003), *Aedes aegypti* (Cheon et al., 2001; Seo et al., 2003) and *Galleria mellonella* (Lee et al., 2003a, b). The insect lipoprotein, lipophorin (Lp; Weers et al., 1993; Babin et al., 1999; Bogerd et al., 2000), was observed to bind to these insect LDLR homologues (Dantuma et al., 1999; Cheon et al., 2001; Seo et al., 2003; Lee et al., 2003a, b). Therefore, the insect lipoprotein receptor is termed lipophorin receptor (LpR). A characteristic feature of Lp is its functioning as a reusable lipid shuttle, i.e. the particle selectively loads and unloads lipids at target tissues without internalization and concomitant degradation (for reviews see Ryan and Van der Horst, 2000; Arrese et al., 2001; Van der Horst et al., 2001, 2002). In apparent contrast to the concept of selective lipid uptake, the occurrence of LpR suggests a fate similar to LDL resulting in complete degradation upon receptor-mediated endocytosis. Therefore, the intriguing question was whether LpR, in contrast to all other LDLR family members, is able to recycle its ligand after internalization.

In our previous studies, CHO cell lines that endogenously express LDLR and TfR were stably transfected with *L. migratoria* LpR cDNA (Van Hoof et al., 2002), and used to analyze the distribution and sorting of internalized mammalian and insect ligands simultaneously. Incubation experiments with fluorescently-labeled ligands showed that Lp is efficiently removed from LDL-containing sorting endosomes after receptor-mediated endocytosis. Both Lp and LpR accumulate in the Tf-positive ERC, and Lp is re-secreted from the cells with a $t_{½}$ of ~13 min. Endocytosis of Lp had been observed earlier in the insect fat body (Bauerfeind and Comnick, 1992; Dantuma et al., 1997; Van Hoof et al., 2003), an organ analogous to mammalian adipose tissue and liver combined (Locke, 1998). Whether fat body cells are able to recycle Lp after endocytosis remained unclear. The fragility and typical composition of fat body tissue render it difficult to allow for quantification of Lp degradation or recycling (Van Hoof et al., 2003).

Transfection of insect cell lines with LpR cDNA to study the intracellular trafficking of Lp could provide an alternative to circumvent experimental problems with fat body tissue. However, little is known about the pathways of ligands that are internalized via receptor-mediated endocytosis in insect cells. Well-defined markers to identify endosomal organelles involved in intracellular transport of internalized proteins in insect cells are not readily available. In the present report we attempted to distinguish

between vesicular compartments involved in lysosomal degradation and those participating in recycling, using *Drosophila* Schneider line 2 (S2) cells transfected with LDLR and TfR cDNA. Fluorescence microscopy analysis showed that incubation of these transfectants with fluorescently-labeled LDL and Tf resulted in colocalization of both ligands in endosomes. In contrast to the segregation of these ligands in mammalian cells, LDLR and Tf were found to colocalize and remain in vesicular organelles for at least 2 h. Although LDL was anticipated to remain in the cell, Tf was expected to be resecreted. Similar to transfected S2 cells, another TfR-transfected insect model cell line (Sf9) did not resecrete Tf after internalization. Additionaly, S2 cells transfected with LpR did not recycle Lp, which is in contrast to our observations in LpR-transfected CHO cells. Taken together, these findings suggest that either insect cells do not possess the ability to resecrete ligands that are recycled in mammalian cells, or that recycling is cell type specific. *L. migratoria* fat body cells that express endogenous LpR appeared to be capable of resecreting Lp after endocytic uptake. Although some vesicles containing fluorescently-labeled Lp remained visible after a 4 h chase, the number of Lp-containing vesicles had significantly decreased, which is indicative of recycling. The vesicular organelles that contained Lp after the chase were larger compared to those immediately after the incubation. This increase in size could be explained by fusion and maturation of endosomes into lysosomes. Consequently, the proposed recycling of Lp in fat body cells may be less efficient than in mammalian cells.

Materials and methods

Antibodies, reagents and proteins

Polyclonal anti-LpR 2189/90 rabbit antibody was raised against a synthetic peptide representing the unique N-terminal 20 amino acids (34-53) of the first cysteine-rich repeat of LpR (Van Hoof et al., 2003); anti-LDLR 121 rabbit antibody (Anderson et al., 1982) was a gift from Drs. Ineke Braakman and Jürgen Gent (Department of Bio-Organic Chemistry, Utrecht University, Utrecht, The Netherlands). Zeocin (InvivoGen), Blasticidin (Invitrogen), Geneticin (G-418) (GibcoBRL), precision protein standards prestained broad range marker (Bio-Rad), alkaline phosphatase-conjugated affinipure goat-anti-rabbit IgG (AP-GAR) and TRITC-conjugated goat-anti-rabbit IgG (TRITC-GAR) (Jackson ImmunoResearch Laboratories Inc.), leupeptin, aprotinin (Sigma), Oregon Green 488 (OG) carboxylic acid, OG-labeled Tf (OG-Tf) and tetramethylrhodamine-labeled Tf (TMR-Tf) (Molecular Probes), BSA and cold water fish gelatin (Sigma) were obtained from commercial sources. Human LDL was isolated from blood plasma (Bloedbank Midden Nederland) as described by Redgrave et al. (1975); Lp was isolated from locust hemolymph by ultracentrifugation as described by Van Hoof et al. (2002).

Cell culture

The S2 (a generous gift from Dr. Marti Bierhuizen, Department of Medical Physiology, UMC Utrecht) and Sf9 (a generous gift from Drs. Moniek Van Oers and Just Vlak, Department of Virology, Wageningen University) cells were cultured in 25 cm^2 polystyrene culture flasks (CellStar) with Insect Xpress (Bio Whittaker) growth medium containing 100 U/ml penicillin G sodium and 100 µg/ml streptomycin sulfate in 85%

saline (Gibco BRL) at 27°C in a humidified atmosphere. Growth medium of cells transfected with pIZ or pIB (Invitrogen) was supplemented with 400 µg/ml Zeocin (Invivogen) and 10 µg/ml Blasticidin (Invitrogen), respectively. For fluorescence microscopy, cells were grown on glass cover slips (∅ 15 mm; Menzel-Gläser) in 12-wells (3.5 cm^2/well) multidishes (Costar), respectively. CHO cells stably expressing *L. migratoria* LpR [CHO(LpR) cells] were cultured as described by Van Hoof et al. (2002).

Construction of insect expression vectors harboring human LDLR and TfR cDNA, and *L. migratoria* LpR cDNA

The pIB plasmid harboring human LDLR cDNA (pIB-LDLR) was constructed by digesting pBS-LDLR (a generous gift from Dr. Ineke Braakman, Department of Bio-Organic Chemistry, Utrecht University, Utrecht, The Netherlands) with *Kpn*I and *Not*I (New England BioLabs), the human LDLR cDNA fragment of which was ligated into pMIB (Invitrogen). This intermediary product was digested with *Hind*III and *Not*I (New England BioLabs) after which the human LDLR cDNA fragment was ligated into pIB (Invitrogen), generating the final pIB-LDLR construct that was used for the transfection of S2 cells.

The plasmids pIZ-TfR and pIZ-LpR were constructed by digesting pCB6-TfR (a generous gift from Dr. Peter Van der Sluijs, Department of Cell Biology, UMC Utrecht, Utrecht, The Netherlands) with *Eco*RI and *Xba*I, and piLR-e (Dantuma et al., 1999) with *Eco*RI and *Not*I, respectively. The generated TfR cDNA and LpR cDNA fragments were ligated into pIZ (Invitrogen) and used for the transfections described below.

Generation of S2 and Sf9 cell lines expressing LDLR, TfR and LpR

Wild-type (wt) S2 and Sf9 cells were grown to ~50% confluency in 25 cm^2 culture flasks (CellStar) and transfected for 5 h with 3 µg of plasmid DNA in 1 ml growth medium supplemented with 20 µl CellFectin reagent (Invitrogen Life Technologies) according to the supplier's protocol. S2 cells were transfected with pIB-LDLR, pIZ-TfR and pIZ-LpR to create the S2(LDLR), S2(TfR), S2(LDLR-TfR) and S2(LpR) cell lines; Sf9 cells were transfected with pIZ-TfR to create the Sf9(TfR) cell line. The cells were transferred to 75 cm^2 culture flasks (CellStar), and grown for 3-4 weeks in selective growth medium, containing 400 µg/ml Zeocin to obtain transfectants stably expressing LDLR, or 10 µg/ml Blasticidin to obtain those stably expressing TfR or LpR. Double transfectants [i.e. S2(LDLR-TfR) cells] were first grown in Zeocin-containing growth medium until the selection was complete. Thereafter, the same cells were cultured in growth medium containing Blasticidin to dispose of cells transfected with only one of the two plasmids. For Lp incubation experiments, wt S2 cells were transiently transfected with LpR cDNA. S2 cells were seeded in 12-well multidishes at 50% confluency, and cultured overnight in a humidified atmosphere at 25-27°C. Transfection was conducted with 0.8 µg pIZ-LpR DNA and 4 µl CellFectin per well according to the supplier's protocol. Approximately 45 h post transfection, S2(LpR) cells were used for incubation experiments as described below.

Western blot analysis of cell membrane extracts

S2 and Sf9 cells were harvested from 25 cm^2 culture flasks (CellStar) at ~90% confluency and resuspended in CHAPS buffer (20 mM HEPES, 124 mM NaCl, 4.7 mM

KCl, 2.5 mM $CaCl_2$, 2.5 mM Na_2HPO_4, 1.2 mM $MgSO_4$, 1 mM EDTA, 0.1 mM benzamidine, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1% CHAPS), incubated for 10 min on ice, and spun down at 15000 *g* for 10 min at 4°C. Supernatants were either heated for 5 min at 95°C in Laemmli buffer (Laemmli, 1970) or directly dissolved in Laemmli buffer without β-mercaptoethanol and 0.025% SDS, prior to separation by SDS-PAGE on a 10% polyacrylamide gel. CHO and fat body cell membrane extracts were used for SDS-PAGE as described by Van Hoof et al. (2002; 2003). The separated membrane proteins were transferred to polyvinylidene fluoride membrane (Millipore) by Western blotting and incubated with anti-LpR 2189/90 rabbit antibody (1:200) or anti-LDLR 121 antibody (1:2000) as indicated for 2 h, followed by 1 h AP-GAR incubation. Hybridized AP-GAR was visualized by incubating the blot in TSM buffer, containing 100 mM Tris-HCl, 100 mM NaCl, 10 mM $MgAc_2$, 50 μg/ml p-nitro blue tetrazolium chloride (NBT; Boehringer Mannheim), 25 μg/ml 5-bromo-4-chloro-3-indoyl-phosphate p-toluidine (BCIP; Roche Diagnostics), pH 9.0.

Incubation of insect cells with fluorescently-labeled ligands

LDL and Lp (1 mg/ml) were fluorescently labeled with 20 μl/ml OG dissolved in DMSO (1 μg/μl) at room temperature under continuous stirring for 1 h according to the manufacturer's instructions. The fluorescently-labeled lipoproteins were purified with Sephadex G-25 PD-10 columns (Amersham Pharmacia Biotech) to replace the PBS by incubation medium containing 10 mM HEPES, 50 mM NaCl, 10 mM KCl, 5 mM $CaCl_2$, 2 mM $MgSO_4$, pH 7.4. For endocytic uptake, stably-transfected S2 and Sf9 cells were seeded in 12-well multidishes at 20% and 30% confluency, respectively, and cultured for 2 days in a humidified atmosphere at 25-27°C. Thereafter, cells were incubated with 35 μg/ml OG-LDL, 25 μg/ml OG-Tf, and 25 μg/ml TMR-Tf as indicated for 15 min at 27°C. Transient transfectants were generated as described above and incubated with 25 μg/ml OG-Lp for 15 min at 27°C. Fat body tissue was excised from young adult *L. migratoria* immediately after final ecdysis and incubated with OG-Lp as described by Van Hoof et al. (2003). Cells were rinsed in incubation medium and either directly fixated in 4% paraformaldehyde diluted in PBS for 30 min at room temperature, or first chased in growth medium at 27°C for variable time periods.

Immunofluorescence

Fixated cells were washed twice with PBS buffer and permeabilized with PBS buffer supplemented with 0.4% Triton X-100 (PBSX) for 5 min at room temperature. The cells were subsequently incubated with PBSX containing 50 mM glycin for 10 min and 5% BSA for 30 min at room temperature. The cells were blocked for 5 min with 0.1% coldwater fish gelatin in PBSX (PBSXG) at room temperature and incubated with corresponding primary antibodies (1:500) for 1 h at 37°C. After rinsing 4 times for 5 min with PBSX and a final wash-step of 5 min in PBSXG at room temperature, the samples were processed for indirect immunofluorescence by incubation with TRITC-GAR for 30 min at 37°C and rinsed an additional 4 times with PBSXG.

Microscopy and image processing

Cover slips with fixated cells were mounted in Mowiol and examined on a fluorescence Axioscop microscope (Zeiss) with a Hg HBO-50 lamp and a Plan-Neofluar 100×/1.30 oil lens. Using FITC/TRITC filters, digital images were acquired with a DXM 1200

digital camera and ACT-1 version 2.00 software (Nikon Corporation). Images were processed using Scion Image beta version 4.0.2 (Scion Corporation) and PaintShop pro 7.00 (Jasc Software) software.

Results

Endocytosis of human LDL and Tf by *Drosophila* S2 cells transfected with human LDLR and TfR

To investigate the functional ligand binding specificity of mammalian LDLR and TfR expressed by insect cells, S2 cells were stably transfected with the insect expression vector pIZ harboring full-length human LDLR or TfR cDNA [S2(LDLR) and S2(TfR), respectively]. Transfected cells were incubated with fluorescently-labeled ligand for 15 min at 27°C in a buffer that was supplemented with HEPES (i.e. incubation medium). Upon incubation with Oregon Green 488 (OG)-labeled human LDL (OG-LDL), a punctate staining pattern was visible, indicative for endocytic vesicles enriched in OG-LDL (Fig. 1A). This pattern was absent in wt S2 cells incubated with OG-LDL (Fig. 1B). LDLR was visualized with immunofluorescence (IF) using the monoclonal antibody α-LDLR C7 raised against the first ligand-binding repeat of LDLR (Anderson et al., 1982), and a TRITC-labeled secondary anti-mouse antibody. Dual labeling studies show that LDL (Fig. 1C) and LDLR (Fig. 1D) colocalize in these endosomes (Fig. 1E).

Similar results were obtained with S2(TfR) cells incubated with tetramethyl-rhodamine (TMR)-labeled Tf (TMR-Tf); incubation of S2(TfR) cells with TMR-Tf resulted in a punctate staining pattern (Fig. 1F) that was absent in wt S2 cells (Fig. 1G). The use of OG-labeled Tf (OG-Tf) allowed us to visualize the colocalization of Tf (Fig. 1H) with TfR (Fig. 1I) in endosomes (Fig. 1J).

Taken together, these findings suggest that shortly after endocytosis, internalized LDL and Tf reside in similar endosomes. To investigate whether LDL and Tf converge in the same endosomes, S2 cells were cotransfected with two vectors harboring cDNA of either human LDLR or TfR. These S2(LDLR-TfR) transfectants expressing both human receptors were incubated with OG-LDL and TMR-Tf, simultaneously. Shortly after incubation, LDL (Fig. 1K) and Tf (Fig. 1L) clearly colocalize (Fig. 1M), which suggests that the two ligands are transferred to the same vesicles after internalization.

Temporal and spatial distrubution of internalized LDL and Tf in transfected S2 cells

In mammalian cells, human LDL is rapidly delivered to sorting endosomes where LDL dissociates from LDLR after which the ligand is degraded in lysosomes and the receptor recycles back to the cell surface. To determine the intracellular fate of LDL after LDLR-mediated endocytosis in insect cells, preincubation of S2(LDLR) cells with OG-LDL was followed by a chase in growth medium without ligand. Cells were fixated at defined time points, and visualized with fluorescence microscopy, which revealed that LDL remains in intracellular vesicular compartments (Fig. 2A-E). Although the number of vesicles per cell gradually decreased, a large amount of LDL was visible for at least 2 h (Fig. 2E).

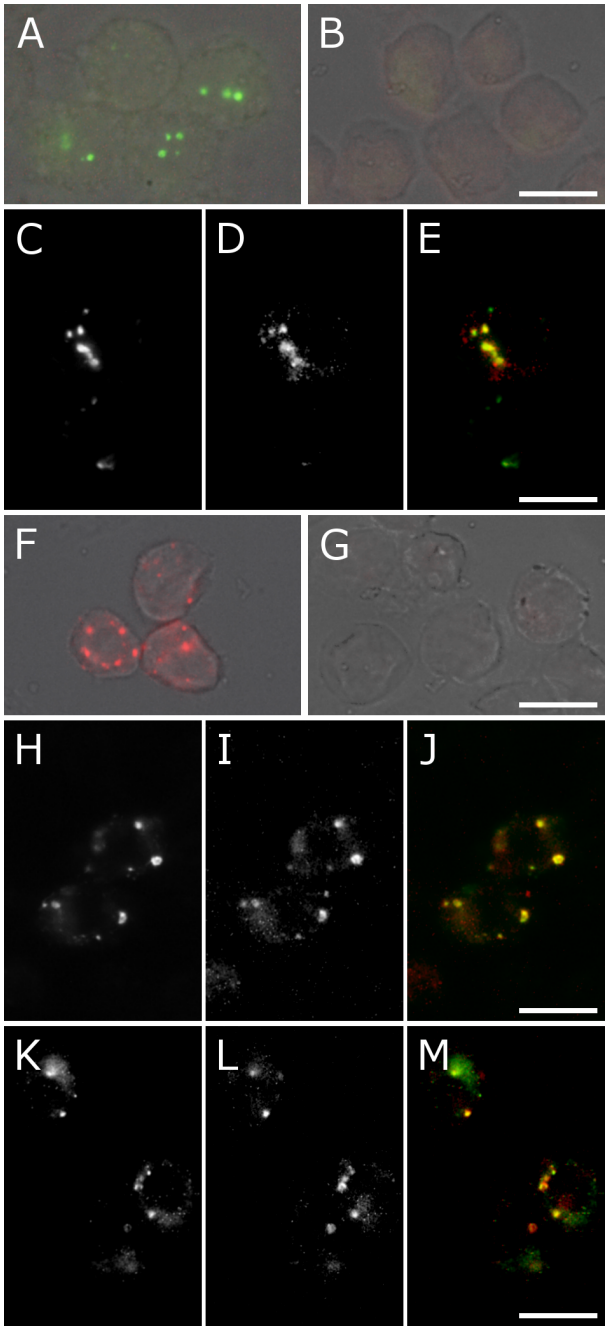

Figure 1. Visualization of receptor-mediated endocytic uptake of fluorescently-labeled LDL and Tf by S2 transfectants using fluorescence microscopy. S2 cells were incubated with OG-LDL in incubation medium for 15 min at 27°C, fixated with paraformaldehyde and either immediately mounted in mowiol (A and B), or after staining the receptors with IF using anti-LDLR C7 mouse antibody (C-E). S2(LDLR) (A) and wt S2 (B) cells were incubated with LDL after which the fluorescence images were overlayed onto transparent bright field pictures of the same cells. The punctate staining pattern in LDLR-transfected cells (A), indicative for receptor-mediated endocytosis of OG-LDL, was absent in wt cells (B). In S2(LDLR) cells that internalize OG-LDL (C), LDLR is recognized by the antibody (D) which colocalizes with the ligand in endosomes (E). A similar staining pattern was observed in S2(TfR) transfectants when incubated with TMR-Tf (F); wt S2 cells did not take up TMR-Tf (G). OG-Tf is also internalized by S2(TfR) transfectants (H) and colocalizes with TfR (I), in endosomes (J). In double transfectants expressing both LDLR and TfR, OG-LDL (K) and TMR-Tf (L) colocalize in endosomes (M) after endocytosis. LDL is shown in green (A, B, E, and M) or grey (C and K); LDLR is shown in grey (D) or red (E); Tf is shown in red (F, G, and M), grey (H and L), or green (J); TfR is shown in grey (I) or red (J); colocalization is shown in yellow (E, J, and M). Bars, 10 μm (B, E, G, J, and M).

Human LDL and Tf follow distinct intracellular routes in mammalian cells. In contrast to LDL, Tf is resecreted after TfR-mediated endocytosis. To address this issue in insect cells, TMR-Tf was used to preincubate S2(TfR) cells after which the

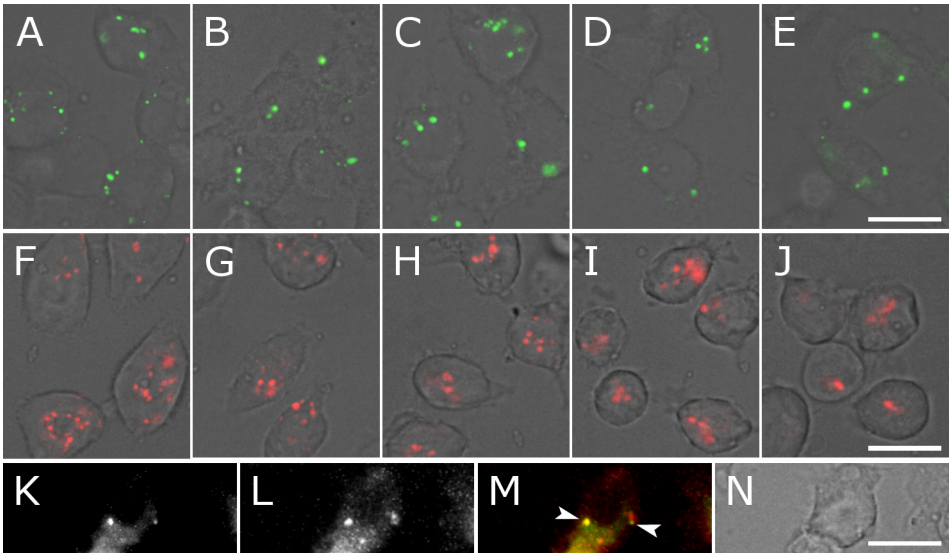

Figure 2. Visualizaton of OG-LDL and TMR-Tf in transfected S2 cells during a chase. S2(LDLR) and S2(TfR) cells were preincubated with OG-LDL (A-E) and TMR-Tf (F-J), respectively, for 15 min at 27°C. Subsequently, the cells were either immediately fixated (A and F), or after a chase in growth medium for 10 (B and G), 30 (C and H), 60 (D and I), or 120 min (E and J). Fluorescence images were overlayed onto bright field pictures of the same cells. S2(LDLR-TfR) double transfectants were preincubated with incubation medium containing OG-LDL and TMR-Tf, and subjected to a chase for 60 min in growth medium (K-M). In transfectants that had internalized OG-LDL (K) as well as TMR-Tf (L), both ligands colocalized in intracellular vesicles (M, arrow heads). A bright field image of the cells in K-M is shown (N). LDL is shown in green (A-E and M) or grey (K); Tf is shown in red (F-J and M) or grey (L); colocalization is shown in yellow (M). Bars, 10 μm (E, J, and N).

incubation medium was replaced by growth medium without ligand. Similar to LDL in S2(LDLR) cells, a 2 h chase did not result in a significant decrease of internalized ligand (Fig. 2F-J). Although, like LDL in S2(LDLR) cells, the number of Tf-containing vesicles decreased to some extent, a considerable proportion of Tf remained in the cells, whereas Tf is resecreted from mamalian cells with a $t_{½}$ of approximately 12 min (Mayor et al., 1993; Ghosh et al., 1994). Moreover, no ERC-like organelle could be identified during any stage of the chase, which suggests that Tf is not recycled in a manner similar to that observed in mammalian cells.

Additional evidence for an identical intracellular fate of LDL and Tf after receptor-mediated endocytosis in insect cells was obtained from pulse-chase experiments using S2(LDLR-TfR) double transfectants. These cells, expressing both receptors simultaneously, were preincubated with incubation medium containing a mixture of OG-LDL and TMR-Tf. When subjected to a chase, LDL (Fig. 2K) and Tf (Fig. 2L) were found to colocalize in endosomal compartments after 2 h (Fig. 2M, arrow heads), which suggests that both ligands end up in the same intracellular vesicles.

Temporal and spatial distrubution of internalized Tf in TfR-transfected Sf9 cells

S2(TfR) cells lack the ability to rapidly resecrete TfR-internalized Tf (Fig. 2F-J), which conflicts with observations of the mammalian system. To determine whether this

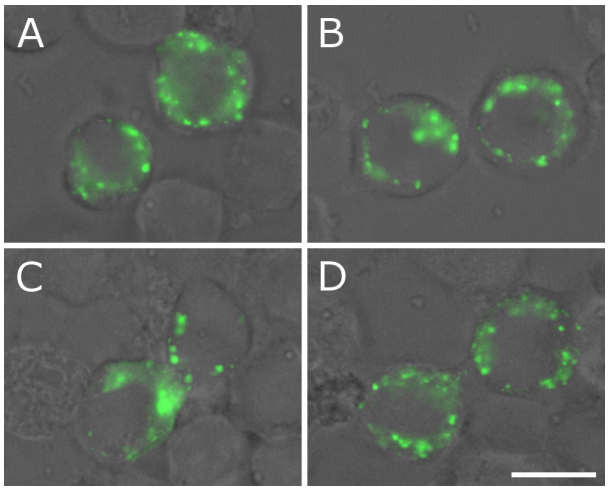

Figure 3. Visualizaton of OG-Tf in Sf9(TfR) transfectants during a chase. Sf9(TfR) cells were preincubated with OG-Tf for 15 min at 27°C and fixated after a chase in growth medium for 10 (A), 30 (B), 60 (C), or 120 min (D). Fluorescence images were overlayed onto bright field pictures of the same cells. Tf is shown in green (A-D). Bar, 10 μm (D).

inability to recycle Tf is specific for S2 cells, we used another insect model cell line. *S. frugiperda* Sf9 cells were stably transfected with human TfR cDNA [Sf9(TfR) cells], using the same expression vector as described for S2(TfR) cells. A chase in growth medium without ligand following preincubation of Sf9(TfR) cells with OG-Tf resulted in a punctate staining pattern (Fig. 3A-D) that was similar to that observed in S2(TfR) cells (Fig. 2F-J). No significant reduction of intracellular Tf was observed, suggesting that these insect cells are unable to recycle the ligand.

Expression of LpR in stably-transfected insect cells

Apparently, mammalian Tf is not recycled in insect cells after TfR-mediated uptake. To investigate whether this is the result of the inability of insect cells to cope with mammalian proteins, we chose an insect receptor that has been shown to efficiently recycle its ligand after receptor-mediated endocytosis in CHO cells (Van Hoof et al., 2002). S2 cells were stably transfected with the insect expression vector harboring *L. migratoria* LpR cDNA [S2(LpR) cells]. The expression of LpR by S2 cells was analyzed using detergent cell extracts that were separated by SDS-PAGE under reducing and non-reducing conditions. The proteins were transferred to polyvinylidene fluoride membrane and immunoblotted with polyclonal anti-LpR 2189/90 rabbit antibody raised against the very N-terminal 20 amino acids of the first ligand-binding repeat (Van Hoof et al., 2003). LpR expressed by S2(LpR) cells was compared to that of stably-transfected CHO cells (Van Hoof et al., 2002) and endogenous LpR expressed by young adult *L. migratoria* fat body tissue (Van Hoof et al., 2003). These Western blots showed that LpR expressed by S2(LpR) cells has an apparent molecular weight of approximately 140 kDa (Fig. 4A, lane 3), which is slightly less than that of LpR produced by CHO cells (~150 kDa; Fig. 4A, lane 1; Van Hoof et al., 2002), and more or less similar to that of endogenous *L. migratoria* LpR (Fig. 4A, lane 2; Van Hoof et al., 2003). The molecular weights of all cell type-specific LpR proteins are higher than the theoretical 98 kDa (Dantuma et al., 1999), which suggests that all receptors are glycosylated. In addition, LpR derived from Sf9 cells transfected with *L. migratoria* LpR cDNA was analyzed on the same Western blot, and appears to have a molecular weight identical to LpR expressed by S2(LpR) cells (Fig. 4A, lane 4). Sf9(LpR) cells

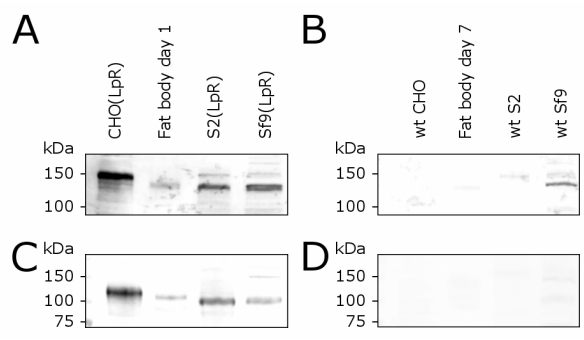

Figure 4. Expression of LpR in transfected cell lines was analyzed on Western blot. (A) Membrane proteins were isolated from CHO(LpR) cells (lane 1), fat body tissue of *L. migratoria* immediately after final ecdysis (lane 2), S2(LpR) cells (lane 3), and Sf9(LpR) cells (lane 4). (B) Membrane proteins were also isolated from wt CHO cells (lane 1), fat body tissue of *L. migratoria* 7 days after final ecdysis (lane 2), wt S2 cells (lane 3), and wt Sf9 cells (lane 4). The samples were denatured for 5 min at 95°C in Laemmli (1970) buffer, and subjected to SDS-PAGE under reducing conditions (A and B). Following transfer to polyvinylidene fluoride membrane, LpR was detected with anti-LpR 2189/90 rabbit antibody. Similar blots were generated under non-reducing conditions using Laemmli buffer containing 0.025% SDS and no reducing agents (C and D). The molecular weight markers (kDa) are indicated on the left of each panel.

produce a double band in the region of 140 kDa, both of which are recognized by anti-LpR 2189/90 antibody under reducing conditions. Only one of those two bands is visible in wt Sf9 cells, which suggests that the other protein in Sf9(LpR) cells is the result of transfection and expression of *L. migratoria* LpR (Fig. 4B, lane 4). No protein was recognized by the antibody in the wt CHO and S2 cells (Fig. 4B, lanes 1 and 3, respectively), or in *L. migratoria* fat body tissue 7 days after final ecdysis (Fig. 4B, lane 2), a stage at which LpR is no longer expressed (Van Hoof et al., 2003).

As observed for LpR derived from CHO(LpR) cells (Fig. 4C, lane 1; Van Hoof et al., 2002) and young adult *L. migratoria* (Fig. 4C, lane 2; Van Hoof et al., 2003), the electrophoretic mobility of LpR from both transfected insect cell lines increased under non-reducing conditions (Fig. 4C, lanes 3 and 4). This coincides with the maintenance of multiple disulfide bonds that are present in the receptor (Dantuma et al., 1999). Like under reducing conditions, no protein was recognized by the antibody in wt CHO and S2 cells (Fig. 4D, lanes 1 and 3, respectively), or day 7 adult fat body tissue (Fig. 4D, lane 2). Although under reducing conditions, a single protein in wt Sf9 cells was specifically recognized by anti-LpR 2189/90 antibody (Fig. 4B, lane 4), a similar band was not detected under non-reducing conditions (Fig. 4D, lane 4). Whereas these findings suggest that the protein has a region homologous to the ligand binding domain of *L. migratoria* LpR, it does not mediate internalization of *L. migratoria* Lp; wt Sf9 cells did not internalize OG-Lp (data not shown).

Temporal and spatial distrubution of internalized Lp in LpR-transfected S2 cells

S2(LpR) and Sf9(LpR) cells were incubated with OG-labeled Lp (OG-Lp) for 15 min at 27°C to test the functional expression of LpR. Whereas Sf9(LpR) transfectants did not internalize Lp (data not shown), the ligand was taken up by S2(LpR) transfectants (Fig. 5A); however, not by wt S2 cells (Fig. 5B). Even though LpR is expressed by both transfected cell lines, and these receptors have a similar electrophoretic mobility under reducing and non-reducing conditions (Fig. 4A and C), the receptor produced by Sf9(LpR) cells appears to be non-functional. After endocytic uptake of OG-Lp, LpR

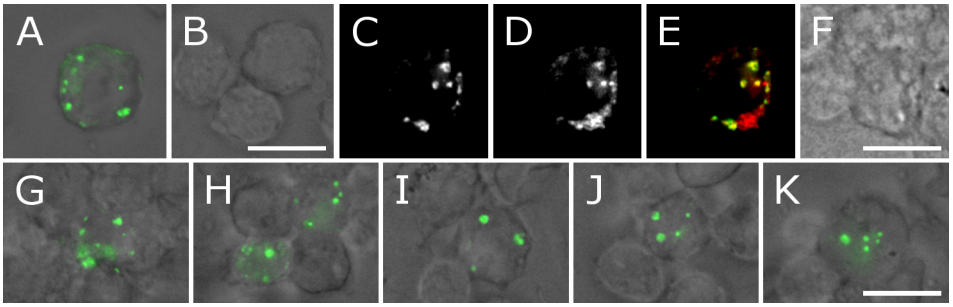

Figure 5. Visualization of receptor-mediated endocytic uptake of OG-Lp by S2(LpR) transfectants. S2 cells were incubated with OG-Lp in incubation medium for 15 min at 27°C, fixated with paraformaldehyde and either immediately mounted in mowiol (A and B), or after staining the receptors with IF using anti-LpR 2189/90 rabbit antibody (C-E). S2(LpR) (A) and wt S2 (B) cells were incubated with Lp after which the fluorescence images were overlayed onto bright field pictures of the same cells. The punctate staining pattern in LpR-transfected cells (A) was absent in wt cells (B). OG-Lp in S2(LpR) cells (C) colocalizes with LpR (D) in endosomes (E). A bright field image of the cells in C-E is shown (F). S2(LpR) cells were preincubated with OG-Lp for 15 min at 27°C, and either either immediately fixated (G), or after a chase in growth medium for 10 (H), 30 (I), 60 (J), or 120 min (K). Fluorescence images were overlayed onto bright field pictures of the same cells. Lp is shown in green (A, B, E, and G-K) or grey (C); LpR is shown in grey (D) or red (E); colocalization is shown in yellow (E). Bars, 10 μm (B, F, and K).

expressed by S2 transfectants was visualized with IF using anti-LpR 2189/90 antibody, showing that Lp (Fig. 5C) and LpR (Fig. 5D) colocalize in endosomes (Fig. 5E). These findings suggest that LpR specifically mediates endocytosis of Lp in S2(LpR) cells. In contrast to the destiny of Lp after internalization by LpR-expressing mammalian cells (Van Hoof et al., 2002), Lp was not significantly resecreted from S2(LpR) cells when subjected to a chase in growth medium for 2 h (Fig 5G-K). Similar to our observations with LDL and Tf in S2 transfectants, the number of vesicles decreased during the chase in comparison to that immediately after incubation. If the decrease of Lp-containing vesicles in S2(LpR) cells indecates recycling of the ligand, the process is not as rapid as observed in LpR-transfected CHO cells.

Temporal and spatial distrubution of internalized Lp in endogenously LpR-expressing *L. migratoria* fat body cells

None of the ligands that recycle in mammalian cells were observed to be rapidly resecreted after receptor-mediated endocytosis in the insect cell lines. The question arises whether this is the result of the type of cells in combination with the receptors and ligands that were used. To investigate whether insect cells possess the ability to recycle ligands after receptor-mediated endocytosis, tissue expressing endogenous LpR was used (Van Hoof et al., 2003). Young adult fat body tissue of *L. migratoria* was preincubated *in vitro* with OG-Lp in incubation medium, and subjected to a chase in insect growth medium without ligand. Latter chase resulted in a significant decrease in the number of OG-Lp-containing vesicles (Fig. 6A-F), indicative of resecretion of the Lp particle after endocytosis. However, these Lp-containing vesicles were larger compared to endosomes immediately after incubation. The increase in vesicle size may be due to fusion and maturation of the endosomes into lysosomes (Stoorvogel et al., 1991; Dunn and Maxfield, 1992); thus, additional degradation of Lp cannot be excluded.

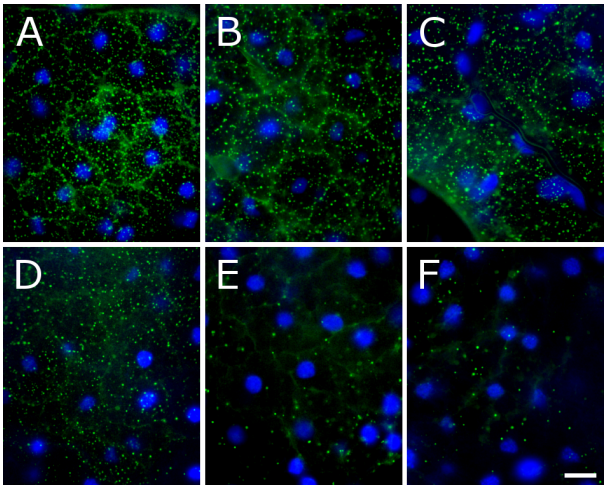

Figure 6. Visualizaton of OG-Lp in *L. migratoria* fat body tissue during a chase. Fat body was excised immediately after final ecdysis, preincubated with OG-Lp for 30 min at 27°C, and either immediately fixated (A), or after a chase in growth medium for 10 (B), 30 (C), 60 (D), 120 (E), or 240 min (F). Nuclei were stained with DAPI after fixation and the pictures were overlaid onto the fluorescence images of OG-Lp. Lp is shown in green (A-F); nuclei are shown in blue (A-F). Bar, 20 μm (F).

Discussion

To elucidate the intracellular trafficking of ligands after receptor-mediated endocytosis in insect cells, *Drosophila* S2 cells were transfected with LDLR and TfR, two well-defined receptors with respect to their distribution of internalized ligands (i.e. LDL and Tf, respectively) in mammalian cells (e.g. CHO cells). In mammalian cells, LDL and Tf follow distinct intracellular routes upon internalization by their receptors. Whereas LDL remains in intracellular vesicles to be degraded in lysosomes (Goldstein et al., 1985; Brown and Goldstein, 1986; Dunn et al., 1989), Tf is efficiently recycled either directly, or via passage through the ERC (Yamashiro et al., 1984; Mayor et al., 1993; Ghosh et al., 1994; Maxfield and McGraw, 2004). In S2(LDLR) cells, LDL was shown to remain in vesicular organelles for at least 2 h (Fig. 2A-E), which suggests that LDL has a similar fate in mammalian and insect cells. Remarkably, Tf was shown to follow a similar intracellular route after endocytosis in S2(TfR) cells (Fig. 2F-J). Instead of being resecreted with a $t_{½}$ of ~12 min, like in mammalian cells (Mayor et al., 2003; Ghosh et al., 1994), Tf remained in vesicular compartments that colocalize with LDL-containing vesicles (Fig. 2K-M). Apparently, the destination of Tf in S2 cells differs from that in mammalian cells. Even though the rate of possible ligand recycling in insect cells may differ from that of mammalian cells, it is unlikely that efficient sorting and subsequent recycling of Tf will occur in vesicles in which LDL also resides after a chase period of 2 h. In addition, Tf was shown to have a similar destination after receptor-mediated uptake in Sf9(TfR) cells (Fig. 3A-D). The absence of an ERC-like organelle containing Tf during a chase in both cell lines also supports a degradative destiny for Tf in insect cells.

Although in insect cells LDL and Tf colocalize in intracellular vesicles that persist for hours (Fig. 2K-M), it is unclear whether these organelles are common degradative lysosomes. The S2 cells used for the experiments are derived from embryonic *Drosophila* tissue (Schneider et al., 1972), and the Sf9 cells from immature ovaries of *S. frugiperda* pupae (Vaughn et al., 1977). Both cell lines are capable of internalizing

hemolymph phospholipoglycoproteins (e.g. vitellogenin) via receptor-mediated endocytosis (e.g. via the vitellogenin receptor) (Raikhel and Lea, 1986). Repetitive fusion of endosomes *in vivo* results in formation of large storage compartments that provide supplies for the developing embryo. Yolk granules constitute the majority of these storage compartments and have an acidic luminal pH (Fagotto and Maxfield, 1994). Even though yolk granules contain lysosomal hydrolases, the constituents are not degraded before late embryogenesis, the stage at which yolk granules are progressively acidified to values below pH 5 (Fagotto, 1995). From this point of view, yolk granules differ from their somatic lysosomal counterparts. Despite these distinct functional characteristics, their common acidity renders it almost impossible to discriminate using standard acidotrophic agents. In addition, the lack of specific antibodies against proteins associated with these typical insect organelles exclude the use of immunofluorescence to mark lysosomes and yolk granules. Possibly, monoclonal transfected cell lines will provide the possibility to quantify the ratios of intact and degraded radioactively-labeled ligands after receptor-mediated endocytosis. Although it would be interesting to determine the identity of the vesicular compartments in insect cells containing both LDL and Tf, it will not give an answer as to why latter protein is not efficiently resecreted from insect cells.

In the past, insect cells have been used to express human LDLR and TfR; albeit not with the goal to study intracellular trafficking of internalized ligands. For instance, the proper expression and localization of several lipoprotein receptors, including human LDLR, on the cell surface of S2 cells were shown to be dependent on boca, a chaperone protein that is endogenously expressed by these cells (Culi and Mann, 2003). No ligand binding or endocytosis experiments were performed with these transfectants. Human TfR has been successfully expressed in developing *Drosophila* oocytes and embryos to study expression and distribution (Bretscher et al., 1996). Human TfR produced in a baculovirus expression system using Sf9 cells, was characterized and shown to be differently glycosylated (Domingo and Trowbridge, 1988). As a consequence, this receptor had a molecular weight smaller than the authentic receptor. Despite the difference in degree of glycosylation, immunofluorescence analysis showed that this TfR occurs at the insect cell surface. Moreover, binding studies with ^{125}I-labeled Tf suggested that the receptor was biologically active. Tf binding studies performed at 28°C gave results similar to those performed at 4°C. It was, therefore, proposed that either most of human TfR is displayed on the cell surface, or that receptors located in intracellular membranes (e.g. upon ligand endocytosis) do not recycle back to the cell surface (Domingo and Trowbridge, 1988). Our findings with fluorescently-labeled Tf (Fig. 2F-J and 3A-D) are in favour of latter hypothesis, which suggests that occupied TfR does not recycle, but remains in the cell. Although under normal conditions this phenomenon does not occur in mammalian cells, it coincides with the biophysical property of TfR not to release bound Tf at low pH. This characteristic feature is most likely independent of the expression system.

Internalized Tf is most likely not dissociated from TfR in endosomes of insect cells due to stability of the complex at low pH. Thus, if Tf remains in intracellular vesicles (Fig. 2F-J), associated TfR is also retained in the cell. This does not exclude other receptors from recycling through insect cells. Possibly, LDL is released from LDLR in endosomes as a result of luminal acidification after which the receptor solitarily returns to the cell surface. Ligand uncoupling after receptor-mediated endocytosis in endosomes of insect cells has been demonstrated in oocytes of the mosquito *A. aegypti*

(Snigirevskaya et al., 1997). Electron microscopy revealed that developing oocytes take up vitellogenin involving the vitellogenin receptor; ligand and receptor colocalized in coated vesicles and early endosomes. These data also showed that the lumen of transitional yolk bodies, analogues of late endosomes in somatic cells, was positively labeled for vitellogenin, whereas the receptor was preferentially localized in the membrane region and tubular extensions of these organelles. In mammalian cells, such tubular extensions are known to bud off from sorting endosomes and recycle back to the plasma membrane (Yamashiro et al., 1984; Dunn et al., 1989; Verges et al., 1999), returning membrane constituents (e.g. transmembrane receptors) to the cell surface (for review see Maxfield and McGraw, 2004). Whereas these tubules in the oocyte were depleted of vitellogenin, yolk granules were heavily labeled for vitellogenin, but completely lacked receptor (Snigirevskaya et al., 1997). Insect cells have also been shown to be capable of recycling human receptors. For example, in transfected Sf9 cells expressing human μ opioid receptor, exposure to ohmefentanyl caused maximum internalization of the receptor at 30 min, and receptors seemed to reappear at the cell membrane after 60 min (Chen et al., 2003). Identification of insect rab proteins provides indirect but strong evidence for the possibility of receptor recycling in insect cells (Satoh et al., 1997). Numerous rab proteins have been analyzed and characterized in mammals and shown to be crucial for the control of vesicular transport in the exocytic, endocytic, and transcytotic pathways (Simons and Zerial, 1993; Novick and Brennwald, 1993). Rab11 plays an essential role in the transport of recycling vesicles that bud off from tubular extensions radiating from sorting endosomes (Mukherjee et al., 1997; Novick and Zerial, 1997; Somsel Rodman and Wandinger-Ness, 2000). This protein has also been found in *Drosophila*, where it was observed to be important for polarization of the cytoplasm in addition to membrane recycling (Dollar et al., 2002). With our fluorescence experiments, it is not possible to determine whether LDLR and TfR are recycled after internalization. As described above, it is most likely that TfR is not recycled when in complex with Tf, but whether this is also the case for LDLR coupled to LDL remains to be investigated. Irrespective of the destination of these receptors upon ligand binding, recycling of internalized unoccupied receptors in insect cells is plausible and cannot be excluded for LDLR, TfR or LpR.

To investigate whether recycling-deficiency of endocytosed Tf in insect cells was due to incompatibility of recycling a mammalian receptor-ligand complex in an insect system, we chose to make use of the insect receptor LpR, which was shown to recycle Lp in stably transfected CHO cells (Van Hoof et al., 2002). As observed in an overexpression system (Fig. 5G-K), LpR failed to completely resecrete Lp in 2 h. These findings suggest that insect cells are either incapable of recycling receptor-bound ligands in general, or do not recycle Lp and foreign ligands such as Tf. In addition to vitellogenin, hemolymph Lp provides developing oocytes with protein and lipid (Kawooya and Law, 1988). Upon internalization by the oocyte, the majority of lipid is unloaded from Lp and stored as triacylglycerol. Similar to vitellogenin, the protein component of Lp is not hydrolyzed, but stored in yolk granules to be degraded later during embryonic development (Kawooya et al., 1988). Therefore, it is not unlikely that Lp is retained in S2 cells, and stored in yolk granules after receptor-mediated endocytosis. The number of Lp-containing vesicles in fat body cells significantly decreased within the first 2 h of the chase (Fig. 6A-E). Although the tissue became not entirely depleted of Lp after a 4 h chase (Fig. 6F), recycling of the ligand could explain the partial evanescence of Lp-containing vesicles. Whether the remaining Lp is

completely degraded, stored intact, or recycled from fat body cells remains obscure. Attempts to quantify Lp uptake and possible recycling in *L. migratoria* fat body tissue had been conducted using ^{125}I-labeled Lp (Dantuma et al., 1997). The results, however, relate to internalization of Lp during a period at which LpR is not significantly expressed. Nevertheless, the assay described in latter study could be used to determine the ratio of intact Lp resecreted after endocytosis by fat body tissue derived from young adults of a stage at which LpR is highly expressed (Van Hoof et al., 2003).

The mechanism via which a cell decides to either recycle or degrade a receptor-ligand complex remains to be elucidated, and apparently depends on the type of cell. In some cases, dissociation of a ligand from its receptor is a prerequisite for recycling of the receptor (Davis et al., 1987). Single point mutations in the LDLR gene affecting its dissociation property irrevocably direct the complex to lysosomes (for review see Hobbs et al., 1992). However, highly homologous receptors (e.g. LpR) that do not uncouple their ligand (e.g. Lp) in sorting endosomes of mammalian cells escape lysosomal degradation, and are transferred to the ERC from which they eventually return to the plasma membrane (Van Hoof et al., 2002). This implies that it is most likely the intracellular tail of the receptor that is exposed in the cytosol via which signals that determine its destination are transferred. We are currently addressing this issue by constructing hybrid receptors of LDLR and LpR in order to obtain clues for the domains that are involved in these processes and responsible for recycling of receptor-ligand complexes.

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CHAPTER 5

Alteration of the intracellular fate of a lipoprotein receptor using a hybrid approach

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Manuscript in preparation

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Alteration of the intracellular fate of a lipoprotein receptor using a hybrid approach

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Abstract

The human low-density lipoprotein receptor (LDLR) mediates ligand endocytosis, and recycles after intracellular ligand dissociation. Mutations in the LDLR gene that impair uncoupling result in degradation of the receptor-ligand complex. The wild-type receptor for insect lipoprotein, lipophorin (Lp), is homologous to LDLR, and also shows impaired uncoupling. However, this lipophorin receptor (LpR) recycles as an LpR-ligand complex after ligand endocytosis *in vitro*. Primary sequence homology analysis of multiple LDLR homologues revealed that putative motifs specific for lipoprotein receptor subfamilies are predominantly located in the intracellular domain. To investigate whether the intracellular tail of LDLR induces degradation of LpR when ligand remains attached, the intracellular C-terminus of insect LpR was replaced by that of human LDLR. This $LpR_{1-790}LDLR_{791-839}$ hybrid receptor internalized and recycled Lp when expressed in Chinese hamster ovary cells. Apparently, the intracellular tail of LDLR does not elicit degradation of a ligand dissociation-deficient lipoprotein receptor. The hybrid approach was extended by generating a receptor harboring the ligand binding domain of LpR and the region from EGF domain to intracellular tail of LDLR. Although ligand endocytosis by this $LpR_{1-342}LDLR_{293-839}$ was unaffected, the receptor did not recycle. These findings suggest that latter hybrid follows an intracellular route that is similar to ligand dissociation-deficient LDLR. In addition, these data imply that the EGF domain and intracellular tail of LDLR collaborate in determining the destination of a receptor-ligand complex.

Introduction

An array of homologous receptors mediates the cellular uptake of lipids derived from a variety of lipid-carrying lipoproteins that circulate through the blood. One of these receptors, the mammalian low-density lipoprotein (LDL) receptor (LDLR), is expressed by the liver and peripheral tissues (for review see Brown and Goldstein, 1986). LDLR is the prototype of this large family of structurally related receptors, and binds LDL (for review see Hussain et al., 1999). LDLR is composed of five characteristic domains (Yamamoto et al., 1984): (1) a ligand binding domain harboring 7 cysteine-rich repeats, (2) an epidermal growth factor (EGF) precursor homology domain comprising two EGF

modules separated from a third by a β-propeller (Jeon et al., 2001), (3) an O-linked glycosylation domain, (4) a transmembrane domain, and (5) an intracellular C-terminal tail. Small members (e.g. LDLR) comprise only one of each of these five domains, whereas large members harbor multiple ligand binding domains and EGF domains in addition to a single O-linked glycosylation domain, transmembrane domain and intracellular tail.

The protein component of LDL consists of one copy of the non-exchangeable apolipoprotein B (apoB; for review see Shelness and Sellers, 2001) that binds to LDLR (for review see Brown and Goldstein, 1986). Very-low-density lipoprotein (VLDL) contains one molecule of apoB in addition to multiple copies of smaller exchangeable apolipoproteins such as apoE (for review see Shelness and Sellers, 2001), the latter of which is also recognized by LDLR (for review see Brown and Goldstein, 1983). The VLDL receptor (VLDLR), another small member of the LDLR family, was found to specifically bind VLDL (for review see Tacken et al., 2001). Instead of being internalized by VLDLR, this ligand is extracellularly depleted of lipids. Whereas VLDLR does not bind LDL, the receptor shows high affinity for receptor-associated protein (RAP; Battey et al., 1994), a chaperone protein that is presumed to assist in the folding of several lipoprotein receptors (for reviews see Bu and Schwartz, 1998; Bu and Marzolo, 2000). In contrast, RAP has no significant affinity for LDLR (Medh et al., 1995). Upon binding to LDLR and VLDLR, ligands are internalized and dissociated from their receptors in tubulo-vesicular sorting endosomes, due to mild acidification of the vesicle lumen (for reviews see Mukherjee et al., 1997; Innerarity, 2002; Jeon and Blacklow, 2003). The receptors enter the tubules that bud off to be transported back to the plasma membrane via the endocytic recycling compartment (ERC; for review see Maxfield and McGraw, 2004), whereas the sorting endosomes mature into lysosomes (Stoorvogel et al., 1991) wherein the luminal constituents (i.e. lipoproteins) are degraded (for reviews see Goldstein et al., 1985; Brown and Goldstein, 1986).

Several small LDLR family members have been identified in insects (Dantuma et al., 1999; Cheon et al., 2001; Seo et al., 2003; Lee et al., 2003a, b). The insect lipoprotein, lipophorin (Lp; Weers et al., 1993; Babin et al., 1999; Bogerd et al., 2000), was observed to bind to these insect LDLR homologues (Dantuma et al., 1999; Cheon et al., 2001; Seo et al., 2003; Lee et al., 2003a, b). Therefore, the insect lipoprotein receptor is termed lipophorin receptor (LpR). *Locusta migratoria* LpR was the first insect LDLR family member cloned (Dantuma et al., 1999) and characterized in a mammalian overexpression system (Van Hoof et al., 2002), as well as in *L. migratoria* fat body tissue (Van Hoof et al., 2003), both in which it binds and internalizes Lp. In contrast to LDL and VLDL, Lp was proposed to remain attached to LpR in sorting endosomes after receptor-mediated endocytosis, and escape lysosomal hydrolysis. Similar to LDLR, LpR is recycled back to the plasma membrane (Van Hoof et al., 2002). As a consequence, Lp is returned to the cell surface, along with LpR.

Numerous mutations in the LDLR gene have been found to affect critical steps of the LDL uptake mechanism, resulting in familial hypercholesterolemia (FH; for reviews see Hobbs et al., 1990, 1992). These mutations have been classified according to the stage at which LDL endocytosis is disturbed. Class 5 encompasses receptors deficient in recycling, which is presumed to be due to the inability of these receptor mutants to release ligand in endosomes (for review see Hobbs et al., 1990). Deletion analysis revealed that the EGF domain is most likely responsible for acid-dependent ligand

dissociation. Although LDL binding was impaired, this LDLRΔEGF was shown to mediate endocytosis of VLDL, after which the receptor-ligand complex was degraded in lysosomes (Davis et al., 1987a). Additional support for the role of the EGF domain in ligand dissociation was obtained from studies using VLDLR, the EGF domain of which was deleted (Mikhailenko et al., 1999). Similar to LDLRΔEGF, this VLDLRΔEGF was shown to be incapable of dissociating ligand. However, in contrast to the LDLRΔEGF-ligand complex (Davis et al., 1987a), the VLDLRΔEGF-ligand complex was recycled (Mikhailenko et al., 1999). This is similar to that of the LpR-ligand complex in mammalian cells (Van Hoof et al., 2002). Despite the identical domain arrangement and high sequence similarity between the three wild-type (wt) receptors discussed above (LDLR, VLDLR, and LpR), their ligands have distinct fates, and destination of receptor as well as ligand vary upon structural alterations. Apparently, the EGF domain is not the only domain that determines the fate of a receptor when in complex with ligand.

Other domains involved in determining the destiny of a lipoprotein receptor impaired in ligand uncoupling could be the glycosylation domain, transmembrane domain, and intracellular tail. The most likely candidate is the intracellular C-terminus that is exposed in the cytoplasm and thus is able to directly interact with cytosolic proteins. Explicit functions have been assigned to other domains that suggest minimal influence in the course of the receptor. The ligand binding domain determines ligand specificity (for review see Hussain et al., 1999), the O-linked glycosylation domain serves as a spacer that provides stability (Davis et al., 1986), and the transmembrane domain anchors the protein in the cell membrane. The EGF domain may play a more prominent, albeit indirect role by creating a receptor condition upon which the fate of the receptor depends. However, the essence of the EGF domain is not conclusive. Absence of this domain in LDLRΔEGF and VLDLRΔEGF induces divergent receptor-ligand complex fates (Davis et al., 1987a; Mikhailenko et al., 1999). Therefore, different motifs in the intracellular tail of LDLR and VLDLR may determine the intracellular pathways of these receptors.

Primary sequence homology analysis that was restricted to the region from O-linked glycosylation domain to intracellular tail revealed the presence of putative motifs specific for members of LDLR subfamilies. Motifs conserved among subfamily members are predominantly located in the intracellular domain. We made use of the unique ligand recycling property of LpR to investigate if the intracellular tail of LDLR induces degradation of LpR when ligand remains attached. A hybrid receptor was constructed, the intracellular *L. migratoria* LpR C-terminus of which was replaced by that of human LDLR. Overexpression of this $LpR_{1\text{-}790}LDLR_{791\text{-}839}$ hybrid receptor in Chinese hamster ovary (CHO) cells revealed that both Lp endocytosis and recycling were unaffected. These results suggest that substitution of the LpR intracellular tail by that of LDLR is not sufficient to generate a receptor that is degraded upon ligand endocytosis. Because the sequence alignment indicated poor conservation of the transmembrane region and O-linked glycosylation domain between LDLR subfamily members, we extended the hybrid approach to the EGF domain. A second hybrid receptor was constructed to address the dichotomy of complex recycling *versus* degradation. This receptor harbored the ligand binding domain of LpR and the region from EGF domain to intracellular C-terminus of LDLR. Similar to $LpR_{1\text{-}790}LDLR_{791\text{-}839}$, this $LpR_{1\text{-}342}LDLR_{293\text{-}839}$ hybrid receptor was able to internalize Lp, however, internalized ligand was not recycled. Moreover, in contrast to $LpR_{1\text{-}790}LDLR_{791\text{-}839}$,

$LpR_{1-342}LDLR_{293-839}$ recycled neither constitutively, nor in complex with Lp. These findings suggest that latter hybrid follows an intracellular route that is similar to the LDLRΔEGF receptor (Davis et al., 1987a).

Materials and methods

Antibodies, reagents and proteins

Polyclonal anti-LpR 2189/90 rabbit antibody was raised against a synthetic peptide representing the unique N-terminal 20 amino acids (34-53) of the first cysteine-rich repeat of LpR (Van Hoof et al., 2003); anti-LDLR C7 mouse antibody (Anderson et al., 1982) was a gift from Drs. Ineke Braakman and Jürgen Gent (Department of Bio-Organic Chemistry, Utrecht University, Utrecht, The Netherlands). Geneticin (G-418) (GibcoBRL), Zeocin (Invivogen), precision protein standards prestained broad range marker (Bio-Rad), alkaline phosphatase-conjugated affinipure goat-anti-rabbit IgG (AP-GAR), TRITC-conjugated goat-anti-rabbit IgG (TRITC-GAR) and TRITC-conjugated goat-anti-mouse IgG (TRITC-GAM) (Jackson ImmunoResearch Laboratories Inc.), leupeptin, aprotinin (Sigma), Oregon Green 488 (OG) carboxylic acid, and OG-labeled Tf (OG-Tf) (Molecular Probes), BSA and cold water fish gelatin (Sigma) were obtained from commercial sources. Human LDL was isolated from blood plasma (Bloedbank Midden Nederland) as described by Redgrave et al. (1975); Lp was isolated from locust hemolymph by ultracentrifugation as described by Van Hoof et al. (2002).

Amino acid sequence homology analysis and phylogenetic tree construction

Lipoprotein receptor amino acid sequences of the species used were obtained with NCBI Entrez Protein search engine (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Protein). Primary sequence alignment was based on the region from O-linked glycosylation domain (starting immediately after the sixth Cys residue of epidermal growth factor homology repeat C) to the very C-terminus. Multiple amino acid sequence alignment of the domain regions was created with the on-line CMBI Clustal-W server (http://www.cmbi.kun.nl/bioinf/tools/clustalw.shtml) using BLOSUM 30 (gap open penalty 10.00, and gap extension penalty 0.05). Some lipoprotein receptors were omitted from the alignment due to low sequence homology of the intracellular domain and the absence of highly conserved motifs. The multiple sequence alignment was manually modified and used to reconstruct an unrooted phylogenetic tree by quartet puzzling and maximum likelihood (Strimmer and Haeseler, 1996), based on 10000 puzzling steps with the on-line software Puzzle (http://bioweb.pasteur.fr/seqanal/interfaces/Puzzle.html), the infile data tree of which was visualized with TreeView 1.6.6 (http://www.taxonomy.zoology.gla.ac.uk/rod/rod.html).

Cell culture

The Chinese hamster ovary (CHO) cells were cultured in 25 cm^2 polystyrene culture flasks (CellStar) with growth medium containing HAM's F-10 Nutrient mixture, 10% heat inactivated fetal bovine serum, 100 U/ml penicillin G sodium and 100 μg/ml streptomycin sulfate in 85% saline (Gibco BRL) at 37°C in a humidified atmosphere of 5% CO_2. Growth medium of cells transfected with pcDNA3 or pcDNA3.1/Zeo(+) plasmid (Invitrogen) was supplemented with 300 μg/ml G-418, or 400 μg/ml Zeocin,

respectively. In addition, 0.2 μM mevalonate was added to growth medium of LDLRΔEGF-transfected cells (Davis et al., 1987b; a generous gift from Dr. Joseph Goldstein, Department of Molecular Genetics, University of Texas Southwestern Medical Center at Dallas, Texas, U.S.A.). For fluorescence microscopy, cells were grown on glass cover slips (∅ 15 mm; Menzel-Gläser) in 12-wells (3.5 cm^2/well) multidishes (Costar), respectively.

Construction of mammalian expression vectors harboring wt LDLR, $LpR_{1-790}LDLR_{791-839}$ and $LpR_{1-342}LDLR_{293-839}$ cDNA constructs

pcDNA3.1/Zeo(+)-LDLR was constructed by subcloning the LDLR cDNA from pBS-LDLR (a generous gift from Dr. Ineke Braakman, Department of Bio-Organic Chemistry, Utrecht University, Utrecht, The Netherlands) to pcDNA3.1/Zeo(+) (Invitrogen) using the restriction enzymes *Kpn*I and *Not*I (New England BioLabs).

pcDNA3-$LpR_{1-790}LDLR_{791-839}$ was made from a combination of PCR fragments amplified with SuperTaq DNA polymerase (Sphaero-Q), and synthetic oligonucleotides (Invitrogen). The plasmid pcDNA3 harboring *L. migratoria* LpR cDNA (piLR-e; Dantuma et al., 1999) contains the two unique restriction sites *Eco*72I and *Cla*I, positioned in the EGF domain and 3'-untranslated region (UTR) of LpR cDNA, respectively. The sequence between these restriction sites was replaced by a double stranded oligonucleotide construct made from a PCR fragment encoding the removed extracellular region and transmembrane domain of LpR, annealed oligonucleotides encoding the intracellular domain of LDLR, and the UTR of LpR from stop codon to the *Cla*I restriction site. The LpR region from Val683 to Arg797 was generated with PCR using primer 5'-CACGTGTATCATCCATATCGACAACC (introduced *Eco*72I site) in combination with primer 5'-CTCGAGTCTTAAGCCGCCAGTTGTAAACTAC CAGAGCCACTATAGC (introduced *Afl*II and *Xho*I sites), and the piLR-e plasmid as template DNA. The PCR fragment was ligated into a pGEM-T vector (Promega), and the resulting plasmid (pGEM-T-$LpR_{683-797}$) was screened for clones without mutations by sequencing. Similarly, the UTR from stop codon to the *Cla*I site was generated with PCR using primer 5'-CTCGAGGTTAGTTCAGTAACTGAC (introduced *Xho*I site) in combination with 5'-ATCGATTATGATTATATTTTGTACTTAATAACC (introduced *Cla*I site) with piLR-e as template DNA. The PCR fragment was ligated into pGEM-T, and the resulting plasmid (pGEM-T-$LpR_{STOP\text{-}ClaI}$) was screened for clones without mutations by sequencing. The PCR fragment in pGEM-T-$LpR_{STOP\text{-}ClaI}$ was subcloned into pGEM-T-$LpR_{683-797}$ after digestion with *Xho*I and *Not*I (New England BioLabs), latter restriction site of which resides in the multiple cloning site of pGEM-T. The resulting intermediary pGEM-T construct contained the region encoding the partial ectodomain and transmembrane domain of LpR (Val683 to Arg797), and the LpR UTR from stop codon to *Cla*I site, which were separated by a short sequence with *Afl*II and *Xho*I sites. This short sequence was substituted by a stretch of annealed synthetic oligonucleotides encoding the intracellular domain of LDLR from Asn791 to stop codon, flanked by sticky ends complementary to *Afl*II and *Xho*I sites. The final product was digested with *Eco*72I and *Cla*I (New England BioLabs) and ligated into piLR-e that was treated with the same restriction enzymes to generate pcDNA3-$LpR_{1-790}LDLR_{791-839}$. The region encoding LDLR intracellular domain was screened for clones without mutations by sequencing.

pcDNA3-$LpR_{1-342}LDLR_{293-839}$ was constructed with two PCR fragments, encoding the ligand binding domain of LpR, and the region from EGF domain to intracellular

C-terminus of LDLR. The LpR ligand binding domain encoding sequence was generated with PCR using primer 5'-GAATTCGGCTTCACGGGAGG (introduced *Eco*RI site) in combination with 5'-CATTCATTGGTACCACATTTTTCTTGTGG (introduced *Kpn*I site), and the piLR-e plasmid as template DNA. The PCR fragment was ligated into pGEM-T, and the resulting plasmid (pGEM-T-LpR_{1-342}) was screened for clones without mutations by sequencing. Similarly, the region encoding from EGF domain to intracellular tail of LDLR was generated with PCR using primer 5'-GCGGTACCAACGAATGCTTGG (introduced *Kpn*I site) in combination with 5'-ATTTAAATTCACGCCACGTCATCCTCC (introduced *Swa*I site) with pBS-LDLR (a generous gift from Dr. Ineke Braakman, Department of Bio-Organic Chemistry, Utrecht University, Utrecht, The Netherlands) as template DNA. The PCR fragment was ligated into pGEM-T, the resulting plasmid (pGEM-T-$LDLR_{293-839}$) was screened for clones without mutations by sequencing. The PCR fragment in pGEM-T-LpR_{1-342} was subcloned into pGEM-T-$LDLR_{293-839}$ after digestion with *Kpn*I and *Sac*II (New England BioLabs), latter restriction site of which resides in the multiple cloning site of pGEM-T. The resulting intermediary construct (pGEM-T-$LpR_{1-342}LDLR_{293-839}$) was digested with *Eco*RI and *Swa*I (New England BioLabs) and ligated into piLR-e that was treated with the same restriction enzymes to generate pcDNA3-$LpR_{1-342}LDLR_{293-839}$.

Generation of CHO cell lines stably expressing $LpR_{1-790}LDLR_{791-839}$ and $LpR_{1-342}LDLR_{293-839}$

Wt CHO cells and CHO cells that do not express functional LDLR (*ldlA*; Kingsley and Krieger, 1984) were grown to ~90% confluency 12-wells multidishes (Costar) and transfected for 16 h with 3 μg of pcDNA3-$LpR_{1-790}LDLR_{791-839}$ or pcDNA3-$LpR_{1-342}LDLR_{293-839}$ plasmid DNA, respectively. Transfection was conducted in 1 ml growth medium supplemented with 5 μl Lipofectamine2000 reagent (Invitrogen Life Technologies) according to the supplier's protocol. After transfection, cells were transferred to 75 cm^2 culture flasks (CellStar), and grown for 10 days in selective growth medium, containing 400 μg/ml G-418 to obtain transfectants stably expressing the hybrid receptors.

Western blot analysis of cell membrane extracts

CHO cells were harvested from 25 cm^2 culture flasks (CellStar) at ~90% confluency and resuspended in CHAPS buffer (20 mM HEPES, 124 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 2.5 mM Na_2HPO_4, 1.2 mM $MgSO_4$, 1 mM EDTA, 0.1 mM benzamidine, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1% CHAPS), incubated for 10 min on ice, and spun down at 15000 *g* for 10 min at 4°C. Supernatants were either heated for 5 min at 95°C in Laemmli buffer (Laemmli, 1970) or immediately dissolved in Laemmli buffer without β-mercaptoethanol and 0.025% SDS, prior to separation by SDS-PAGE on a 10% polyacrylamide gel. CHO cell membrane extracts were used for SDS-PAGE as described by Van Hoof et al. (2002). The separated membrane proteins were transferred to polyvinylidene fluoride membrane (Millipore) by Western blotting and incubated with anti-iLR 2189/90 rabbit antibody (1:200) for 2 h, followed by 1 h AP-GAR incubation. Hybridized AP-GAR was visualized by incubating the blot in TSM buffer, containing 100 mM Tris-HCl, 100 mM NaCl, 10 mM $MgAc_2$, 50 μg/ml p-nitro blue tetrazolium chloride (NBT; Boehringer Mannheim), 25 μg/ml 5-bromo-4-chloro-3-indoyl-phosphate p-toluidine (BCIP; Roche Diagnostics), pH 9.0.

Incubation of cells with fluorescently-labeled ligands

Lp (1 mg/ml) was fluorescently labeled with 20 µl/ml OG dissolved in DMSO (1 µg/µl) at room temperature under continuous stirring for 1 h according to the manufacturer's instructions. Fluorescently-labeled lipoprotein was purified with Sephadex G-25 PD-10 columns (Amersham Pharmacia Biotech) to replace the PBS by incubation medium containing 10 mM HEPES, 50 mM NaCl, 10 mM KCl, 5 mM $CaCl_2$, 2 mM $MgSO_4$, pH 7.4. For endocytic uptake, cells were seeded in 12-well multidishes at 20% confluency, and cultured for 2 days in a humidified atmosphere at 37°C. Thereafter, cells were incubated with 25 µg/ml OG-Lp, or 25 µg/ml OG-Tf, 25 µg/ml as indicated for 15 min at 37°C. After incubation, cells were rinsed in incubation medium and either immediately fixated in 4% paraformaldehyde diluted in PBS for 30 min at room temperature, or first chased in growth medium at 37°C as indicated.

Immunofluorescence

Immunofluorescence (IF) was used to visualize receptors overexpressed in the transfectants. Fixated cells were washed twice with PBS buffer and permeabilized with PBS buffer supplemented with 0.4% Triton X-100 (PBSX) for 5 min at room temperature. The cells were subsequently incubated with PBSX containing 50 mM glycin for 10 min and 5% BSA for 30 min at room temperature. The cells were blocked for 5 min with 0.1% cold water fish gelatin in PBSX (PBSXG) at room temperature and incubated with corresponding primary antibodies (1:500) for 1 h at 37°C. After rinsing 4 times for 5 min with PBSX and a final wash-step of 5 min in PBSXG at room temperature, the samples were processed for indirect IF by incubation with TRITC-labeled secondary antibody for 30 min at 37°C and rinsed an additional 4 times with PBSXG.

Fluorescence microscopy and image processing

Cover slips with fixated cells were mounted in Mowiol and examined on a fluorescence Axioscop microscope (Zeiss) with a Hg HBO-50 lamp and a Plan-Neofluar 100×/1.30 oil lens. Using FITC/TRITC filters, digital images were acquired with a DXM 1200 digital camera and ACT-1 version 2.00 software (Nikon Corporation). Images were processed using Scion Image beta version 4.0.2 (Scion Corporation) and PaintShop pro 7.00 (Jasc Software) software.

Quantification of receptor recycling efficiency was conducted by incubating the cells with OG-Lp as described, after which the receptor was visualized with IF. The amount of cells in which the receptors had perceptibly converged in the ERC was divided by the total number of cells that were capable of internalizing OG-Lp. Each data set was the result of a duplicate experiment, except when indicated.

Three-dimensional modeling of the LpR ectodomain

The on-line program Swiss-Model (http://swissmodel.expasy.org/) was used to generate a three-dimensional reconstruction of the LpR ectodomain (Guex and Peitsch, 1997; Schwede et al., 2003). Alignments LpR domains were made of regions from Gln128 to Cys205 and Pro206 to Ala773 with PDB entry 1N7D (http://www.rcsb.org/pdb/index.html) as template using the First Approach mode. These alignments were manually optimized, and used as input data for construction of a three-dimensional

model based on the elucidated crystal structure of the LDLR ectodomain at pH 5.3 (Rudenko et al., 2002). The modeled LpR domains were superpositioned onto the structure of LDLR using Swiss PDB Viewer 3.6b3 software.

Results

Primary sequence homology analysis of the region from O-linked glycosylation domain to intracellular C-terminus of LDLR family members

To obtain clues for the presence of motifs that determine the fate of a lipoprotein receptor after ligand internalization, amino acid sequences of several LDLR family members from a variety of species were compared (Table 1). The predominant roles of ligand binding domain and EGF domain are presumed to mediate ligand binding and ligand dissociation, respectively. Thus, the primary sequence homology analysis was restricted to the region from O-linked glycosylation domain to intracellular tail of the receptors.

The alignment shows that the O-linked glycosylation domain is conserved to some extent between similar receptors from different species; however not between different receptors from the same species (Fig. 1, left of the dark grey box with white characters). The transmembrane domain is rich in hydrophobic amino acid residues and functions as a membrane anchor. Despite a common enrichment in Ala, Val, Leu and Ile, this region is poorly conserved between the receptors (Fig. 1, dark grey box with black characters),except for a Pro. The N-terminus of the transmembrane region is not obvious from the amino acid sequence. However, its C-terminus is assumed to end before the W/YK/RNW motif (Killian, 2003), which is highly conserved (Fig. 1, first black box). Assuming that the general length of a transmembrane domain comprises approximately 20 amino acid residues, the Pro acts as a break in the second turn of the α-helix. The corresponding position in LpR is occupied by a Gly or Ser. Like Pro, these residues are poor α-helix formers (Branden and Tooze, 1998), and most likely have a function similar to Pro. In contrast, such spacer residue is absent from the transmembrane domain of the two *C. elegans* receptors, as well as from human, mouse and rat LDLR-related protein (LRP)-2. In addition to the FD/ENPxY (where x represents any amino acid) internalization motif (Fig. 1, third black box), several other highly conserved sequences are found in the intracellular domain of all family members (Fig. 1, second, fourth, and fifth black box), the functions of which are speculative. Putative phosphorylation sites are universally present in the intracellular tail of lipoprotein receptors. Some supposedly other motifs are restricted to receptors that belong to the same subfamily, e.g. the putative PDZ domain interacting motif (for reviews see Sheng and Sala, 2001; Hung and Sheng, 2002) at the C-termini of LpR, LRP-2, and the *C. elegans* receptors (Fig. 1, light grey boxes with black characters). Some of these sequences may be important for intracellular trafficking of the receptor-ligand complex.

The alignment data was used to reconstruct an unrooted phylogenetic tree by quartet puzzling with maximum likelihood (Strimmer and Haeseler, 1996) whose branch lengths are proportional to the degree of relative sequence divergence (Fig. 2). Even though the structure of the tree is not based on homology between domains of the

Table 1. Vertebrate and invertebrate LDLR family members.

Species	Receptor	Abbreviation	Accession #
Homo sapiens (human)	LDLR	Hm LDLR	NP_000518
	VLDLR	Hm VLDLR	NP_003374
	LRP	Hm LRP	NP_002323
	LRP-2	Hm LRP2	NP_004516
	LRP-8	Hm LRP8	NP_004622
Mus musculus (house mouse)	LDLR	Mm LDLR	QRMSLD
	VLDLR	Mm VLDLR	P98156
	LRP	Mm LRP	NP_032538
	LRP-2	Mm LRP2	XP_130308
	LRP-8	Mm LRP8	NP_444303
Rattus norvegicus (Norway rat)	LDLR	Rn LDLR	NP_786938
	VLDLR	Rn VLDLR	NP_037287
	LRP-2	Rn LRP2	NP_110454
Oryctolagus cuniculus (rabbit)	LDLR	Oc LDLR	P20063
	VLDLR	Oc VLDLR	P35953
Sus scrofa (pig)	LDLR	Ss LDLR	AAC39254
Cricetulus griseus (Chinese hamster)	LDLR	Cg LDLR	P35950
Bos taurus (cow)	VLDLR	Bt VLDLR	NP_776914
Gallus gallus (chicken)	LRP	Gg LRP	P98157
	LR8B	Gg LR8B	CAA65729
	VgR	Gg VgR	P98165
Danio rerio (zebrafish)	LDLR	Dr LDLR	AAP22970
Chiloscyllium plagiosum (whitespotted bambooshark)	LDLR	Cp LDLR	AAB42184
Oreochromis aureus (blue tilapia)	VgR	Oa VgR	AAO27569
Oncorhynchus mykiss (rainbow trout)	VgR	Om VgR	CAD10640
	SLR	Om SLR	CAA05874
Xenopus laevis (African clawed frog)	LDLR	Xl LDLR	Q99087
	VLDLR	Xl VLDLR	JC4858
Drosophila melanogaster (fruit fly)	GH26833p	Dm GH26833	AAQ22563
	RE38584p	Dm RE38584	AAN71387
Locusta migratoria (migratory locust)	LpR	Lm LpR	CAA03855
Galleria mellonella (wax moth)	LpR	Gm LpR	n/a
Aedes aegepti (yellow fever mosquito)	LpR	Aa LpR	AAK72954
Anopheles gambiae (African malaria mosquito)	LpR	Ag LpR	XP_308000
Caenorhabditis elegans (nematode)	LRP-1	Ce LRP1	NP_492127
	RME-2	Ce RME2	NP_500815

* The sequence was obtained from Lee et al. (2003a).

receptors that determine ligand specificity (i.e. the ligand binding domain), or the conserved EGF domains, it reflects the generally accepted phylogeny of evolutionary relationship between the lipoprotein receptors. Lipoprotein receptors previously subdivided according to number of cysteine-rich repeats and domain arrangement converge in groups with strong reliability support (70-97%). In addition, vitellogenin receptors (Schneider, 1996; Sappington and Raikhel, 1998a) and VLDLRs clustered into a clade (reliability 70%), which suggests that both family members have evolved from a common recent ancestor. LpRs form an isolated branch that diverges from other lipoprotein receptors, except the two *D. melanogaster* receptors. This indicates that LpRs constitute a unique group of lipoprotein receptors that evolved independently

from the other family members. Although the phylogenic tree implies that *C. elegans* receptors branch from LRP and LRP-2, the length of both branches denotes a distant relationship.

Expression of $LpR_{1-790}LDLR_{791-839}$ and $LpR_{1-342}LDLR_{293-839}$ hybrid receptors in transfected CHO cells

To study the role of the intracellular tail of LDLR in receptor-ligand complex degradation, an LpR-LDLR hybrid receptor was constructed. The intracellular tail of LpR was replaced by that of human LDLR ($LpR_{1-790}LDLR_{791-839}$; Fig. 3A). The hybrid

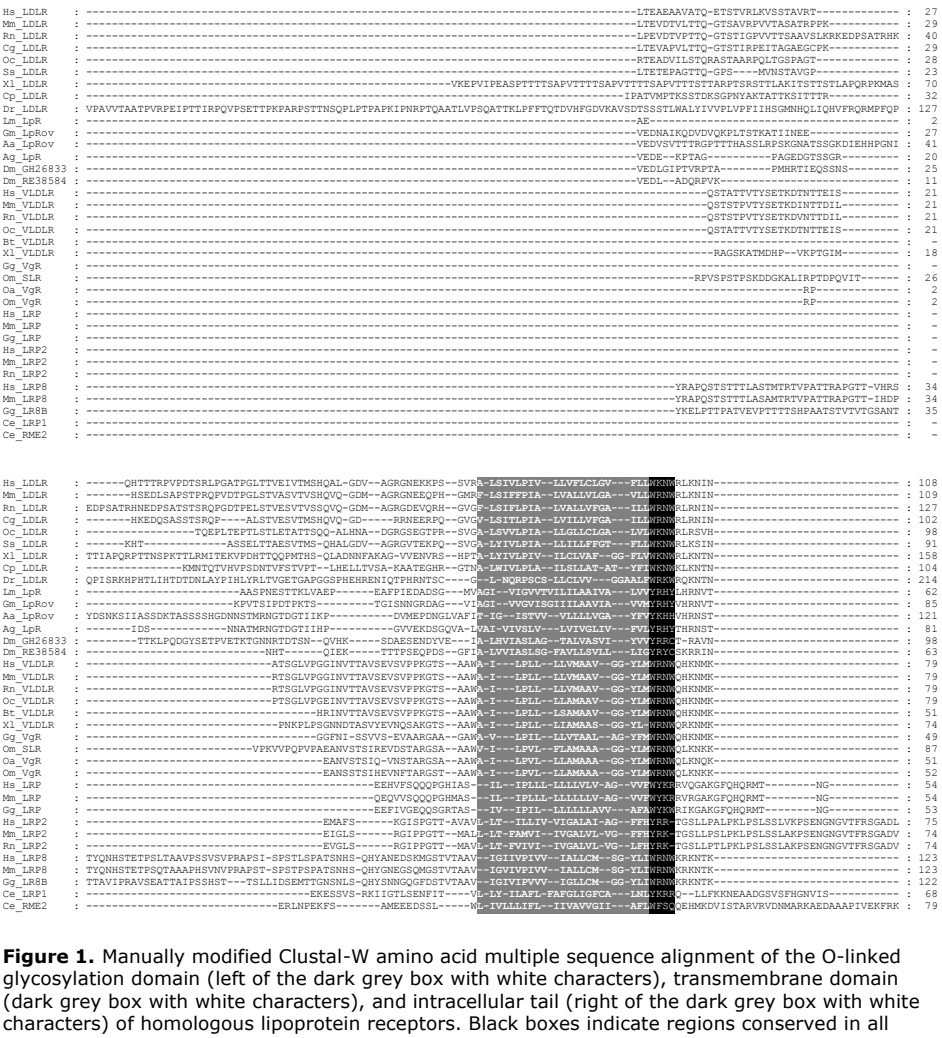

Figure 1. Manually modified Clustal-W amino acid multiple sequence alignment of the O-linked glycosylation domain (left of the dark grey box with white characters), transmembrane domain (dark grey box with white characters), and intracellular tail (right of the dark grey box with white characters) of homologous lipoprotein receptors. Black boxes indicate regions conserved in all receptors. The putative PDZ domains specifically conserved in LpR, LRP-2 and *C. elegans* receptors are marked with a light grey box with black characters. See Table 1 for abbreviations of lipoprotein receptors.

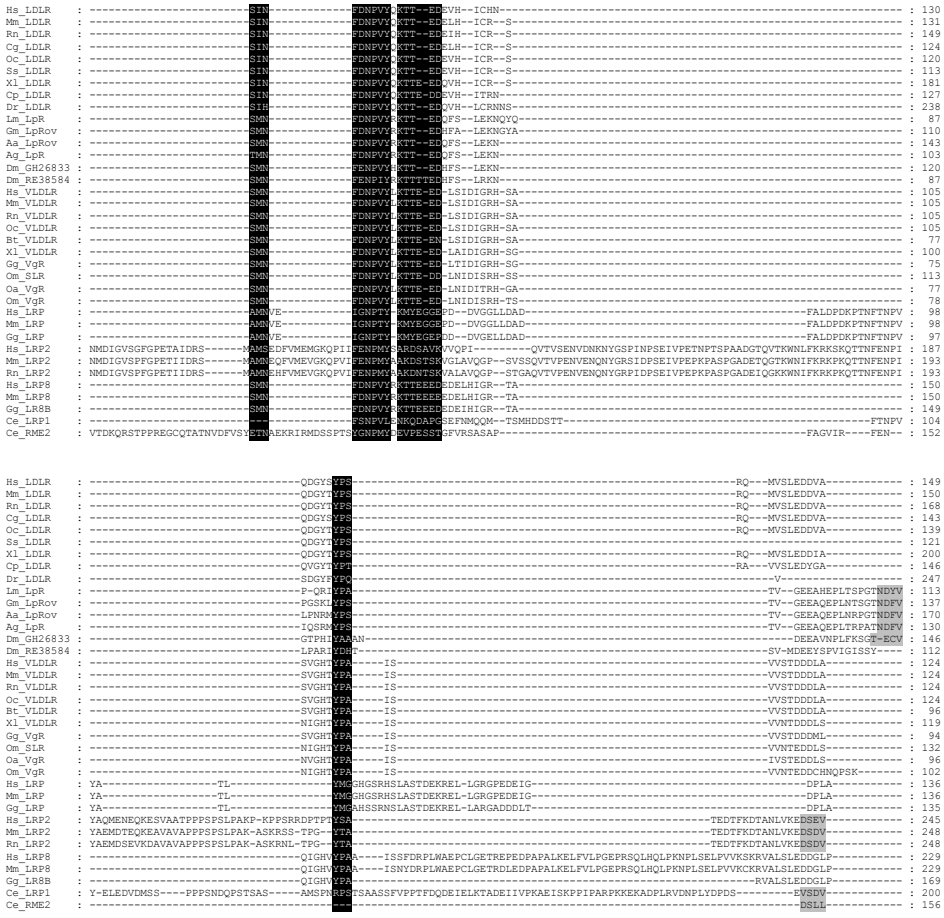

Figure 1. Continued.

approach was extended with a second hybrid receptor to investigate the involvement of the EGF domain in the process of recycling *versus* degradation. This receptor harbored the ligand binding domain of LpR, and the region from EGF domain to intracellular C-terminus of LDLR ($LpR_{1-342}LDLR_{293-839}$; Fig. 3A). CHO cells were stably transfected with the mammalian expression vector pcDNA3 harboring either the $LpR_{1-790}LDLR_{791-839}$ or $LpR_{1-342}LDLR_{293-839}$ recombinant DNA construct, generating the polyclonal cell lines CHO($LpR_{1-790}LDLR_{791-839}$) and CHO($LpR_{1-342}LDLR_{293-839}$), respectively. The expression of hybrid receptor by both transfected cell lines was analyzed using detergent cell extracts that were separated by SDS-PAGE under reducing and non-reducing conditions. The proteins were transferred to polyvinylidene fluoride membrane and immunoblotted with polyclonal anti-LpR 2189/90 rabbit antibody raised against the very N-terminal region of the ligand binding domain of LpR. These Western blots show that in addition to wt LpR, the antibody specifically recognizes both hybrid receptors (Fig. 3B and C). Under reducing conditions, the molecular weights of LpR (Fig. 3B,

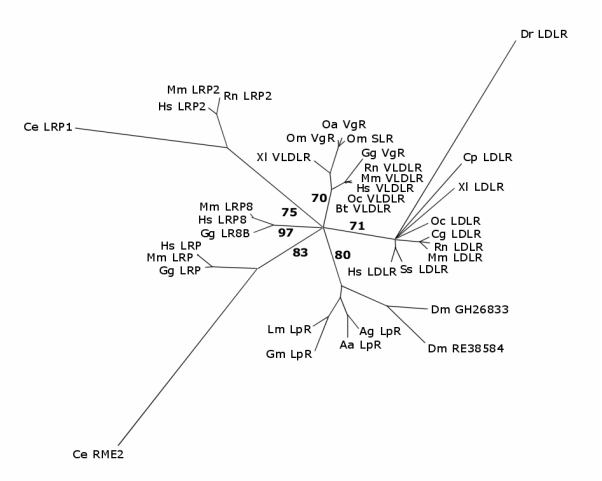

Figure 2. Phylogenic reconstruction by quartet puzzling (Strimmer and Haeseler, 1996) of lipoprotein receptor regions from O-linked glycosylation domain to intracellular C-terminus, based on 10000 puzzling steps. Unrooted tree with reliability values (numerals) that indicate in percent how often the corresponding cluster was found among the 10000 intermediate trees. See Table 1 for abbreviations of lipoprotein receptors. Branch lengths are proportional to the degree of relative sequence divergence.

lane 2; Van Hoof et al., 2002) and $LpR_{1-790}LDLR_{791-839}$ (Fig. 3B, lane 3) are ~150 kDa, whereas $LpR_{1-342}LDLR_{293-839}$ is ~160 kDa (Fig. 3B, lane 4). The difference in molecular weight between $LpR_{1-342}LDLR_{293-839}$ and the other two receptors coincides with the presence of the LDLR O-linked glycosylation domain, which is most likely more heavily glycosylated than that of LpR (Davis et al., 1986). The electrophoretic mobility under reducing conditions (Fig. 3B) is lower than under non-reducing conditions (Fig. 3C), which is consistent with the presence of multiple disulfide bonds in the ligand binding domain and EGF domain. The receptors behave similarly under non-reducing conditions, suggesting that all receptors are folded correctly and functionally expressed on the cell surface.

Endocytosis and intracellular trafficking of Lp in CHO($LpR_{1-790}LDLR_{791-839}$) and CHO($LpR_{1-342}LDLR_{293-839}$) cells

To test the transfected cells for functional expression of hybrid receptors, we incubated the cells for 15 min at 37°C in buffer supplemented with HEPES (incubation medium), containing Oregon Green 488 (OG)-labeled Lp (OG-Lp). Lp specifically binds to LpR (Van Hoof et al., 2002, 2003), and is assumed to attach to the ligand binding domain. Non-transfected wt CHO cells are unable to endocytose the ligand and remain devoid of fluorescently-labeled vesicles upon incubation (Fig. 4A). In wt LpR-transfected cells, OG-Lp accumulates in endosomes that can be visualized with fluorescence microscopy (Fig. 4B; Van Hoof et al., 2002). A vesicle staining pattern identical to that of LpR-transfected cells was observed in CHO($LpR_{1-790}LDLR_{791-839}$) cells after OG-Lp incubation (Fig. 4C). Visualization of the hybrid receptor with IF using the polyclonal anti-LpR rabbit antibody revealed that in most of the cells, the receptor was concentrated in an organelle localized near the nucleus (Fig. 4D and E). Similarly, CHO($LpR_{1-342}LDLR_{293-839}$) cells efficiently internalized Lp (Fig. 4F). However, in the majority of functionally transfected cells, the hybrid receptor was not eminently localized in this juxtanuclear organelle (Fig. 4G and H).

LpR was observed to recycle constitutively in CHO cells (Van Hoof et al., 2002). Human transferrin (Tf), a ligand that is known to efficiently recycle via the ERC upon Tf receptor-mediated endocytosis (Yamashiro et al., 1984), colocalizes with wt LpR in

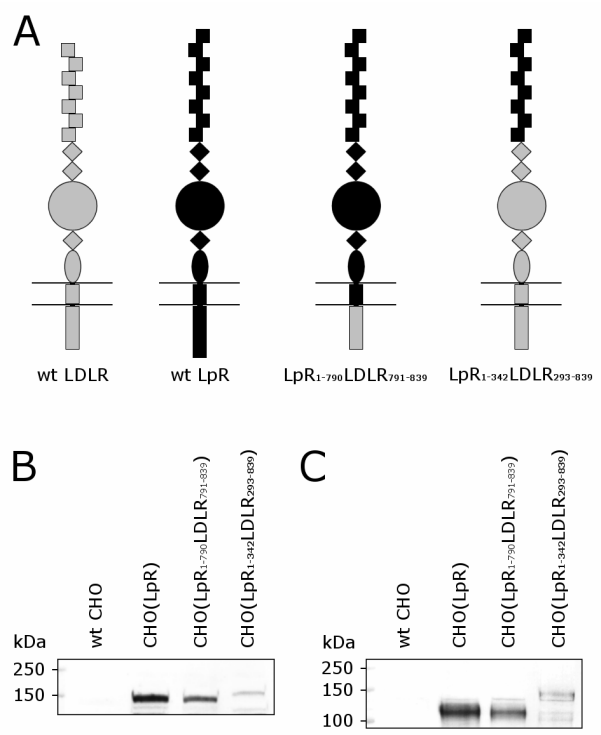

Figure 3. Schematic models of wt LDLR, wt LpR, $LpR_{1-790}LDLR_{791-839}$, and $LpR_{1-342}LDLR_{293-839}$; LDLR domains are depicted in grey, and LpR domains in black (A). LDLR is composed of a ligand binding domain harboring 7 cysteine-rich repeats (squares), an EGF domain comprising two consecutive EGF repeats (diamonds) that are separated from a third by a β-propeller (circle), an O-linked glycosylation domain (oval), a transmembrane domain (short rectangle), and an intracellular tail (long rectangle). The domain arrangement of LpR is identical to LDLR; however, the ligand binding cluster of LpR contains 8 cysteine-rich repeats, and the intracellular tail is longer. $LpR_{1-790}LDLR_{791-839}$ is composed of the complete ectodomain and transmembrane domain of LpR, and the intracellular tail of LDLR. $LpR_{1-342}LDLR_{293-839}$ harbors the ligand binding domain of LpR, whereas its region from EGF domain to intracellular C-terminus is derived from LDLR. (B and C) Expression of receptors in transfected CHO cell lines was analyzed on Western blot. Membrane proteins were isolated from wt CHO cells (lane 1), and cells transfected with wt LpR (lane 2), $LpR_{1-790}LDLR_{791-839}$ (lane 3), or $LpR_{1-342}LDLR_{293-839}$ (lane 4) as described in the materials and methods section. Samples were either denatured for 5 min at 95°C in Laemmli (1970) buffer (B), or dissolved in Laemmli buffer containing 0.025% SDS and no reducing agents, and directly subjected to SDS-PAGE under non-reducing conditions (C). Following transfer to polyvinylidene fluoride membrane, wt LpR, $LpR_{1-790}LDLR_{791-839}$, and $LpR_{1-342}LDLR_{293-839}$ were detected with anti-LpR 2189/90 rabbit antibody. The molecular weight markers (kDa) are indicated on the left of each panel.

the ERC (Van Hoof et al., 2002). After incubation of wt LpR-transfectants with OG-labeled Tf (OG-Tf) for 15 min at 37°C, followed by a 10 min chase in growth medium without ligand, OG-Tf (Fig. 4I) colocalizes with wt LpR (Fig. 4J) that has converged in the ERC (Fig. 4K). In contrast to $LpR_{1-790}LDLR_{791-839}$, which also colocalizes with Tf (Fig. 4L-N), $LpR_{1-342}LDLR_{293-839}$ hybrid receptors did neither converge in a juxtanuclear organelle, nor colocalize with Tf under similar conditions (Fig. 4O-Q).

Latter findings suggest that $LpR_{1-342}LDLR_{293-839}$ is not efficiently recycled. As a consequence, internalized Lp is most likely degraded instead of resecreted after receptor-mediated endocytosis by $LpR_{1-342}LDLR_{293-839}$. To investigate this, the cells were subjected to a chase for 60 min at 37°C in growth medium depleted of OG-Lp following receptor-mediated endocytosis as described above, and analyzed for the presence of intracellularly-remaining ligand. CHO($LpR_{1-790}LDLR_{791-839}$) cells showed a significant reduction of OG-Lp-containing vesicles after the chase (Fig. 4R), and the hybrid receptor (Fig. 4S) was predominantly found in the ERC in most of the cells (Fig. 4T). This suggests that, similar to LpR (Van Hoof et al., 2002), Lp follows the same route as the hybrid receptor, and is eventually secreted from the ERC. Although a

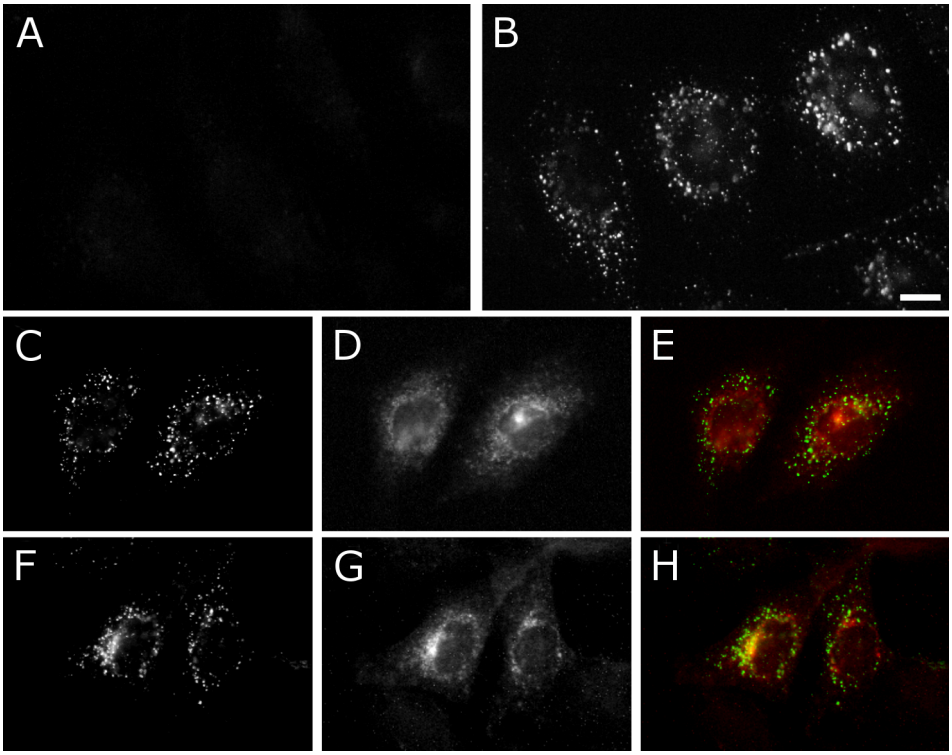

Figure 4. Receptor-mediated endocytic uptake of fluorescently-labeled ligands by wt and transfected CHO cells. Cells were incubated with OG-labeled ligand in incubation medium for 15 min at 37°C, fixated with paraformaldehyde, and either immediately mounted in mowiol (A and B), or after staining the receptors with IF using anti-LpR rabbit antibody (C-W). In contrast to wt cells (A), fluorescence microscopy reveals a punctate staining pattern in LpR-transfected cells (B), which is indicative for receptor-mediated endocytosis of OG-Lp. A similar staining pattern was observed in $LpR_{1-790}LDLR_{791-839}$ transfectants (C); in most of these cells, the receptor was prominently present in the juxtanuclear area (D), which did not contain ligand (E). Although $LpR_{1-342}LDLR_{293-839}$-transfected cells showed a particulate ligand pattern (F), the receptors neither converged (G), nor colocalized with internalized ligand (H). Incubation with OG-Tf, followed by a short chase of 10 min in growth medium showed that Tf accumulates in the ERC (I), the organelle in which wt LpR (J) is also localized (K; Van Hoof et al., 2002). Tf (L) also colocalizes with $LpR_{1-790}LDLR_{791-839}$ (M) in the ERC (N). In contrast, no colocalization of Tf (O) and $LpR_{1-342}LDLR_{293-839}$ (P) in the ERC (Q) was observed. When subjected to a chase for 60 min in the absence of ligand after preincubation with OG-Lp, no ligand was discernable in $LpR_{1-790}LDLR_{791-839}$ transfectants (R), whereas the receptor was prominently localized in the ERC (S and T). Although the number of vesicles and fluorescence intensity were reduced in comparison to immediately after ligand incubation (F), Lp was visible in vesicles after 60 min chase (U), and did not signifiantly colocalize with $LpR_{1-342}LDLR_{293-839}$ (V and W). Lp is shown in grey (A-C, F, R, and U) or green (E, H, T, and W); the receptor is shown in grey (D, G, J, M, P, S, and V) or red (E, H, K, N, Q, T, and W); Tf is shown in grey (I, L, and O) or green (K, N, and Q); colocalization is shown in yellow (E, H, K, N, Q, T, and W). Bars, 10 μm (B and W).

reduction of Lp was observed in CHO($LpR_{1-342}LDLR_{293-839}$) cells, a significant amount of ligand remained in intracellular vesicles (Fig. 4U), and the ERC was devoid of hybrid receptor in the majority of the cells (Fig. 4V and W). Taken together, these findings imply that Lp is recycled in transfected CHO cells after receptor-mediated endocytosis by either LpR or $LpR_{1-790}LDLR_{791-839}$; however, not after internalization mediated by $LpR_{1-342}LDLR_{293-839}$. In addition, they suggest that LpR and $LpR_{1-790}LDLR_{791-839}$ behave

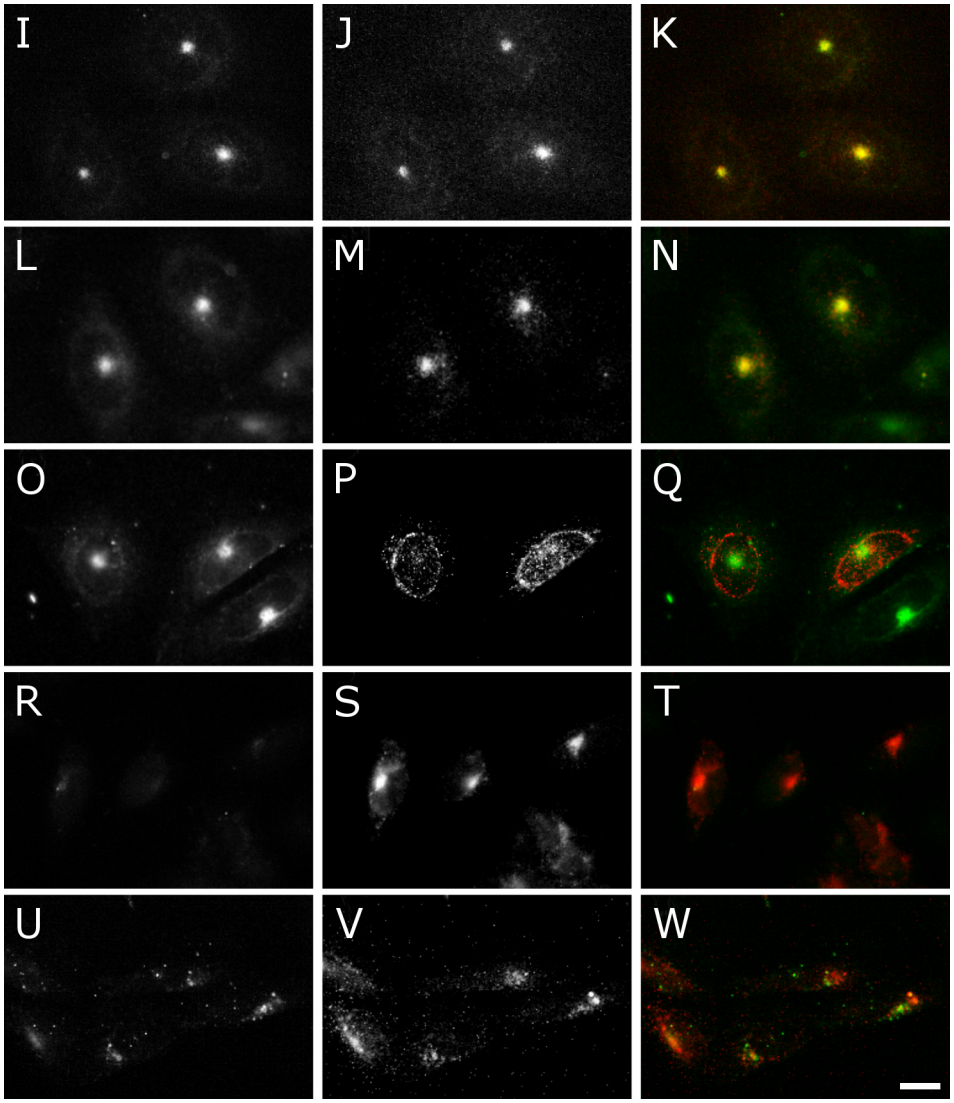

Figure 4. Continued.

similarly in CHO cells, whereas the intracellular fate of $LpR_{1-342}LDLR_{293-839}$ corresponds to that of recycling-deficient LDLR (Davis et al., 1987a).

To determine whether the hybrid receptors have the ability to recycle efficiently, the relative number of transfectants in which the receptors perceptibly converged in the ERC was assessed after a 15 min pulse with fluorescently-labeled Lp. IF revealed that in 56.2% of CHO($LpR_{1-790}LDLR_{791-839}$) transfectants that had internalized OG-Lp, the receptor was prominently localized in the ERC (Table 2). This is more or less equal to that observed in CHO cell lines transfected with wt LDLR (57.2%), and only slightly lower than wt LpR (67.2%) (Table 2); both receptors are known to recycle

Table 2. Evaluation of the percentage of transfectants with a discernable amount of receptor in the ERC, indecative for constitutive receptor recycling.

Receptor	Percentage positive cells
LDLR	57.2% (SD=1.8%, n=482)
LpR	67.2% (SD=4.2%, n=617)
$LpR_{1-790}LDLR_{791-839}$	56.2% (SD<0.1%, n=407)
$LpR_{1-342}LDLR_{293-839}$	19.5% (SD=2.4%, n=1101)
LDLRΔEGF	16.7% (single experiment, n=384)

constitutively (Basu et al., 1981; Anderson et al., 1982; Van Hoof et al., 2002). In contrast, receptor recycling efficiency in CHO($LpR_{1-342}LDLR_{293-839}$) cells was approximately 3-fold lower (19.5%), and similar to that in LDLRΔEGF transfectants (16.7%), which strongly suggests that these receptors are unable to recycle efficiently.

Discussion

Normally, LDLR removes circulating LDL, which contains a relatively high amount of cholesterol and cholesteryl esters in comparison to other lipoproteins (for review see Shelness and Sellers, 2001). LDL is removed by binding to LDLR and internalization of receptor-ligand complex into the cell (for reviews see Goldstein et al., 1985; Brown and Goldstein, 1986). Thus, the proper functioning of LDLR is essential for plasma cholesterol homeostasis. After receptor-mediated endocytosis, LDL is uncoupled from LDLR and degraded in lysosomes whereas the receptor is transported back to the cell surface. This is the default pathway for membrane-anchored proteins (Dunn et al., 1989; Mayor et al., 1993; Verges et al., 1999). Naturally occurring mutations in the LDLR gene that affect the functioning of LDLR result in FH, and may eventually lead to atherosclerosis (for reviews see Hobbs et al., 1990, 1992). Single point mutations in the EGF domain are sufficient to render the receptor incapable of releasing LDL after receptor-mediated endocytosis (for review see Hobbs et al., 1990, 1992). Because deletion of the EGF domain generated a receptor that was unable to release bound ligand (VLDL), it was suggested that this domain is essential for receptor-ligand complex dissociation (Davis et al., 1987a). *In vitro*, the stable complex was observed to be completely degraded in lysosomes, a process that *in vivo* dramatically reduces the number of available cell surface receptors that normally last for ~150 cycles (for review see Goldstein et al., 1979). The role of the EGF domain in ligand dissociation was confirmed by studies with the VLDLR, the EGF domain of which was also removed. However, in contrast to the fate of LDLRΔEGF-ligand complex, the complex between VLDLRΔEGF and ligand, for which RAP was used, was recycled (Mikhailenko et al., 1999). Moreover, the insect LDLR family member, LpR, was observed to recycle in complex with ligand in a mammalian expression system without deletion of the EGF domain (Van Hoof et al., 2002), suggesting that this wt receptor is dissociation deficient. Therefore, the intriguing question was what receptor domain determines the fate of a dissociation-deficient lipoprotein receptor.

Some domains (e.g. the EGF domain) have been shown to play an essential role in determining the pathway of a lipoprotein receptor. The fate of a receptor-ligand complex is receptor-specific, and most likely depends on more than one receptor

domain. The process of ligand dissociation from the ligand binding domain is mediated by the EGF domain, and appears essential for recycling of LDLR. Communication of the EGF domain with the rest of the cell about the status of the receptor most likely involves the domains via which this domain is connected to the cell interior. Because the intracellular C-terminus is exposed in the cytosol, it can directly interact with proteins in the cytoplasm, and transmit signals obtained from the receptor ectodomain. Therefore, this domain is likely to be important for directing the course of the receptor. In addition to the intracellular tail, the transmembrane and O-linked glycosylation domain may also participate in the process. We aligned these sequences of multiple LDLR family members from different species, some of which were omitted from the alignment due to the absence of several key amino acid motifs, like the FD/ENPxY internalization signal (Bansal and Gierasch, 1991). The O-linked glycosylation domain sequence homology is moderate between similar receptors from different species (Fig. 1, left of the dark grey box with white characters), suggesting the presence of sequence motifs that are specific for subfamilies. Nevertheless, deletion of this domain from LDLR did not alter its endocytic and recycling functions (Davis et al., 1986). The transmembrane domain is only poorly conserved (Fig. 1, dark grey box with white characters). However, a single helix-breaking amino acid residue (e.g. Pro) appears to be present in almost all family members. Although a Pro usually produces a significant bend if it is in the middle of an α-helix, it fits well in the first turn of a helix (Branden and Tooze, 1998). In contrast to the transmembrane domain, many highly conserved yet uncharacterized motifs become apparent from the alignment of intracellular C-termini (Fig. 1, right of the dark grey box), which may have signaling functions (Herz and Bock, 2002; Schneider and Nimpf, 2003). Whereas several motifs are universally present in almost every receptor (e.g. W/YK/RNW, SM/IN, FD/ENPxY, KTTED/ED/E and YPS/A; Fig. 1, black boxes), others are amino acid stretches unique for a subfamily. Possibly, some represent motifs with receptor-specific functions, like the putative PDZ domain interacting motif (for reviews see Sheng and Sala, 2001; Hung and Sheng, 2002) at the very C-terminus of LpR (NDF/YV), LPR-2 (DSD/EV), and *C. elegans* receptors (V/DSD/LV/L) (Fig. 1, light grey boxes with black characters). Motifs involved in the determination of the receptor-ligand complex fate are not immediately obvious from the alignment; however, might be identified with mutational analysis of amino acid sequences unique in LDLR.

Common categorization of LDLR family members defined by the number of EGF domains, ligand binding domains, and cysteine-rich repeats within such clusters is problematic because of the repetitive nature of these modules that are sensitive to rearrangements, deletions, and duplications during evolution. Successful attempts have been made with fingerprinting individual cysteine-rich repeats by identifying relatively conserved, as well as unique amino acid residues within the clusters (Sappington and Raikhel, 1998b). This sophisticated method requires specialized software, and does not take into account the domains that are less susceptible to rearrangements. Restricting the alignment to sequences from O-linked glycosylation domain to intracellular tail (Fig. 1) generates a tree, the structure of which accurately represents the generally accepted subdivision of related receptors based on their overall domain arrangement, primary sequence, and ligand binding capacity (Fig. 2). This tree was constructed with the alignment data of domains that are less sensitive to changes. Therefore, it may represent the evolutionary relationship of lipoprotein receptors more accurately than trees based on the overall domain arrangment similarity, and primary sequence homology, as well

as the number of cysteine-rich repeats per ligand binding domain and their ligand specificity. The tree also clearly shows that LpRs form a unique subfamily of lipoprotein receptors, despite the number of cysteine-rich repeats in their single ligand binding domain being identical to that of VLDLR. In addition, although the two *C. elegans* receptors branch from LRP and LRP-2, they both appear to be distantly related to eachother as well as the other members of the family. These data support the assumption that the modern lipoprotein receptors found in vertebrates and insects may have evolved from primordial receptors such as those found in the nematode.

Most investigations on lipoprotein receptors involve ligand binding specificity of the ligand binding domain. The main interest has been focused on intravesicular ligand release; the initial step that determines the intracellular pathway of the receptor-ligand complex (Davis et al., 1987a; Mikhailenko et al., 1999; Van Hoof et al., 2002; Rudenko et al., 2002). These studies indicate the absence of a consensus about the fate of a dissociation-deficient receptor. Thus far, LDLR is the only lipoprotein receptor observed to be degraded when the receptor-ligand complex remains intact (Davis et al., 1987a). Extensive mutational analysis conducted on the LDLR intracellular tail suggested that the very C-terminal 28 amino acids are neither required for internalization, nor for recycling (Davis et al., 1987b). The intracellular tail is essential for efficient endocytosis, and may be important for regulatory functions that are not apparent in cultured cells. In addition to participation in signal transduction pathways (Herz and Bock, 2002; Schneider and Nimpf, 2003), the intracellular domain is presumed to interact with clathrin and other cytosolic proteins (e.g. FE65 and mammalian disabled, Trommsdorff et al., 1998; ARH, He et al., 2002; SNX17, Burden et al., 2004). Therefore, we suspected that this domain of LDLR also induces degradation of the LDLR-ligand complex when dissociation is impaired. Substitution of the intracellular tail of LpR by that of LDLR did not result in expression of a recycling-deficient hybrid receptor (Fig. 4D and H). However, when all domains of LpR, with the exception of the ligand binding domain, were replaced, the resulting hybrid receptor had completely lost the ability to recycle (Fig. 4F an J). These findings suggest that the intracellular route of a stable receptor-ligand complex is not solely determined by the intracellular C-terminus, but depends on the combination of intracellular tail and EGF domain. Apparently, the domains located on either side of the membrane have to be able to communicate either intramolecularly, or via intermediary proteins. Whereas wt LpR recycles, $LpR_{1\text{-}342}LDLR_{293\text{-}839}$ is not predominantly localized in the ERC. Thus, signals transmitted by the ectodomain, and recognized by the intracellular domain are most likely receptor type-specific.

The EGF domain is presumed to play a major role in the intricate process of acid-dependent ligand dissociation (Davis et al., 1987a, Mikhailenko et al., 1999). This process is essential for LDLR recycling (Davis et al., 1987a), however, it is not a prerequisite for LpR to be transferred to the ERC (Van Hoof et al., 2002). Despite the presence of an EGF domain, neither Lp, nor RAP was dissociated from the ligand binding domain of LpR in the endosomal lumen of transfected CHO cells (Van Hoof et al., 2002), which contradicts with the proposed function of the EGF domain. Acid-induced ligand uncoupling is presumed to be the result of a conformational change of the receptor ectodomain. The crystal structure of the ligand binding domain and EGF domain reveals that at low pH, the ligand binding domain is folded onto the β-propeller of the EGF domain (Rudenko et al., 2002), the latter of which thereby occupies the binding site for LDL (for reviews see Innerarity, 2002; Jeon and Blacklow, 2003).

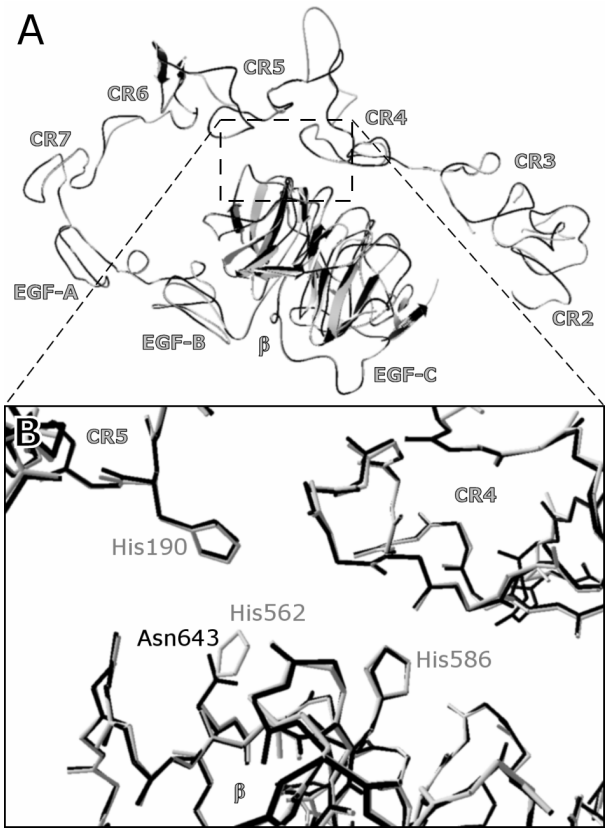

Figure 5. His562 in LDLR corresponds to Asn643 in LpR. (A and B) Superposition of a three-dimensional model of the LpR (black) ectodomain from the third cysteine-rich repeat to the EGF-C module onto the elucidated crystal structure of the LDLR ectodomain at pH 5.3 (grey). (A) Ribbon model of the backbone structure of LDLR and LpR after superposition. (B) Detail of the dashed frame in (A), showing side chains of His190, His562, and His586 of LDLR (grey) in the interface between cysteine-rich repeat 4 and 5 of the ligand binding domain and the β-propeller of the EGF domain in LDLR, and the Asn643 residue in LpR (black). Cysteine-rich repeats of LDLR are indicated with **CR** (grey); EGF repeats are indicated with **EGF** (grey); β-propeller is indicated with b (grey).

LDLR-expressing cells incubated with LDL at 4°C to prevent endocytosis release receptor-bound LDL when washed in an acidic buffer (Brown et al., 1982). This suggests that intramolecular interactions between the ligand binding domain and EGF domain that occur during acidification of the vesicle lumen do not require assisting proteins, and that the conformational change of a lipoprotein receptor is independent of the expression system. Thus, similar interactions between the ligand binding domain and EGF domain of LpR are either not established, or do not involve the docking site of Lp. Because Lp was shown to have no significant affinity for LDLR (Van Hoof et al., 2002), and the $LpR_{1-342}LDLR_{293-839}$ hybrid was able to bind Lp, the docking site of Lp is assigned to the ligand binding domain, as this is the only part of LpR that is present in this hybrid receptor.

A total of three His residues that become protonated below pH 6.7 are present in the interface between the EGF domain and ligand binding domain of LDLR (Rudenko et al., 2002). His190 resides in the fifth cysteine-rich repeat of the ligand binding domain, and His562 and His 586 are located in the β-propellor of the EGF domain. Superposition of a three-dimensional model of LpR (Fig. 5A) onto the elucidated structure of the LDLR ectodomain at pH 5.3 (Rudenko et al., 2002) reveals that only two of the three His residues in LDLR are present in LpR; His562 corresponds to Asn643 of LpR (Fig. 5B). Two independent naturally occurring mutations in LDLR

result in a similar substitution of this residue (H562Y), which lead to FH (Sun et al., 1994; J.C. Defesche, 2003, personal communication). Taken together, these findings suggest that, wt LpR lacks an essential His residue in the EGF domain that corresponds to His562 in LDLR, which participates in the process of ligand dissociation. It would, therefore, seem plausible that the $LpR_{1-342}LDLR_{293-839}$ hybrid is able to dissociate Lp and recycle afterwards; however, this is not the case (Fig. 4I and J). The fluorescence microscopic data suggest that the ligand remains attached to the receptor, and that the receptor-ligand complex is not returned to the cell surface. Apparently, multiple residues are essential to establish as well as maintain a folded receptor conformation that evokes ligand release. The construction of additional hybrids of LDLR and LpR, as well as point mutations of amino acid residues participating in sustenance of the ligand binding domain-EGF domain interface of LDLR at low pH will contribute to the understanding of ligand dissociation and subsequent recycling of lipoprotein receptors in general.

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CHAPTER 6

VI

Divergent effects of familial hypercholesterolemia class 5 LDL receptor mutations: impaired ligand dissociation or defective receptor recycling

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Manuscript in preparation

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Divergent effects of familial hypercholesterolemia class 5 LDL receptor mutations: impaired ligand dissociation or defective receptor recycling

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Abstract

Low-density lipoprotein (LDL) receptor (LDLR) removes cholesterol-rich LDL from the blood by endocytosis. Normally, the epidermal growth factor (EGF) homology domain of LDLR displaces bound LDL in endosomes; whereas LDL is transferred to lysosomes, LDLR recycles back to the cell surface. Familial hypercholesterolemia (FH) class 5 LDLR mutations are located in the LDLR EGF domain and impair intracellular ligand dissociation. Deletion of the EGF domain generates a receptor (LDLRΔEGF) that does not recycle *in vitro*, and results in FH *in vivo*. We studied the naturally occurring LDLR H562Y ($LDLR_{H562Y}$) FH mutation *in vitro*, which showed constitutive recycling; the receptor, however, was degraded after LDL internalization. Even though the insect lipophorin receptor (LpR) bears a high homology to LDLR, it recycles both without and in complex with bound ligand. Tertiary structure analysis shows that His562 in LDLR corresponds to Asn643 in LpR; yet, $LDLR_{H562N}$ did not recycle in complex with ligand, but behaves like $LDLR_{H562Y}$. Substitution of the ligand binding domain of LpR by that of LDLR generates a hybrid receptor ($LDLR_{1-292}LpR_{343-850}$) that is able to bind LDL, indicating that the LDLR EGF domain is not essential for LDL binding. Similar to both LDLR mutants, $LDLR_{1-292}LpR_{343-850}$ recycles constitutively, and is degraded after LDL internalization. The data suggest that class 5 mutations can be divided in two distinct subclasses: (1) mutations that impair ligand dissociation (e.g. $LDLR_{H562Y}$), and (2) those that prevent receptor recycling (e.g. LDLRΔEGF). Both types of subclasses most likely lead to increased degradation of LDLR *in vivo*, resulting in FH.

Introduction

Low-density lipoprotein (LDL) is one of the lipoproteins that transport lipids through the circulation (Frayn, 1996; Ganong, 2001; Jonas, 2002). In comparison to other lipoproteins, LDL carries a relatively high concentration of cholesterol and cholesteryl esters. The particle is taken up from the blood via receptor-mediated endocytosis involving the LDL receptor (LDLR; for review see Brown and Goldstein, 1986). LDLR is the prototype of a large family of structurally related lipoprotein receptors (for review

see Willnow, 1999; Hussain et al., 1999) and is composed of 5 typical domains (Yamamoto et al., 1984): (1) a ligand binding domain, (2) an epidermal growth factor (EGF) precursor homology domain, (3) an O-linked glycosylation domain, (4) a transmembrane domain, and (5) an intracellular C-terminus. Numerous naturally occurring mutations in the LDLR gene lead to familial hypercholesterolemia (FH; for reviews see Hobbs et al., 1990, 1992). FH mutations in the LDLR gene disable LDLR function, and are subdivided in 5 different classes. Class 5 mutations result in the production of recycling-defective LDLR. These mutant receptors are able to bind and internalize LDL, however, do not return to the cell surface after endocytosis.

Normally, LDLR is internalized and recycled either with or without antecedent ligand binding and has a life span of ~150 cycles of endocytosis (for review see Goldstein et al., 1979). It is proposed that class 5 mutations prevent LDLR to recycle properly (for review see Hobbs et al., 1992), resulting in a reduction of the number of LDL-binding surface receptors. As a consequence, cholesterol accumulates in the blood, and may eventually lead to increased incidence of premature ischemic heart disease and tendon xanthomas. Many class 5 mutations are located in the EGF domain, which is proposed to play a crucial role in intracellular ligand dissociation (Davis et al., 1987). Deletion of this domain from LDLR generated a receptor (LDLRΔEGF) that was unable to release its ligand in the endosome upon acidification of the vesicle lumen *in vitro* (Davis et al., 1987); its fate *in vivo* is assumed to be identical (Miyake et al., 1989). Recently, elucidation of the crystal structure of the LDLR ectodomain revealed that at low pH, the ligand binding domain is folded onto the β-propeller of the EGF domain (Rudenko et al., 2002). From the reconstructed three-dimensional model, it became apparent that essential His residues, which are protonated below pH 6.7, establish bridges in the interface between the ligand binding domain and EGF domain. It was proposed that *in vivo*, the EGF domain serves as an alternative ligand for the ligand binding domain that displaces LDL from latter domain due to an increased affinity of the EGF domain for the ligand binding domain upon acidification of the endosomal milieu (for reviews see Innerarity, 2002; Jeon and Blacklow, 2003).

Support for the ligand dissociating function of the EGF domain was obtained from studies with the very-low-density lipoprotein receptor (VLDLR), the domain arrangement of which is identical to that of LDLR (Takahashi et al., 1992). Although endogenously expressed VLDLR is presumed to function extracellularly (for review see Tacken et al., 2001), the receptor was shown to mediate endocytosis and intracellular dissociation of ligands in a mammalian overexpression system (Takahashi et al., 1992). When the EGF domain of this receptor was removed, the resulting VLDLRΔEGF was no longer able to uncouple bound ligand after endocytosis (Mikhailenko et al., 1999). Instead, the ligand was observed to be translocated to the endocytic recycling compartment (ERC), via which internalized membrane constituents (e.g. LDLR and VLDLR) are returned to the plasma membrane (for review see Maxfield and McGraw, 2004).

The first LDLR family member from insects was identified and sequenced in *Locusta migratoria* (Dantuma et al., 1999). The insect lipoprotein, lipophorin (Lp; Weers et al., 1993; Babin et al., 1999; Bogerd et al., 2000), holds an apolipoprotein component homologous to that of LDL (Babin et al., 1999; Mann et al., 1999; Segrest et al., 2001). Lp was observed to bind specifically to the insect LDLR homologue (Dantuma et al., 1999; Van Hoof et al., 2002). Therefore, this insect receptor is termed

lipophorin receptor (LpR). LpR was shown to mediate binding and endocytosis of Lp in insect fat body tissue endogenously expressing LpR (Van Hoof et al., 2003). In contrast to LDLR and VLDLR, LpR efficiently recycles internalized ligand when overexpressed in Chinese hamster ovary (CHO) cells (Van Hoof et al., 2002). Although LpR harbors an EGF domain, which is homologous to that of LDLR, it seems incapable of intravesicular ligand dissociation *in vitro*. Tertiary structural analysis revealed that one of the His residues in the β-propeller of human LDLR (His562) is not present in LpR; instead, LpR contains and Asn at the corresponding position. Two mutations found in independent families resulting in substitution of the LDLR residue His562 by Tyr were clinically diagnosed as FH class 5 mutations (Sun et al., 1994; J.C. Defesche, 2003, personal communication). It was unknown whether these naturally occurring mutations behave like the ligand-recycling wild-type (wt) LpR, or have a lysosomal destination similar to the LDLRΔEGF deletion mutant.

In a previous investigation, we studied the intracellular pathway of a hybrid receptor composed of the ligand binding domain of LpR and the region from EGF domain to intracellular C-terminus from LDLR ($LpR_{1-342}LDLR_{293-839}$). These *in vitro* observations suggested that introduction of an EGF domain harboring His562 in addition to the other proposed essential His residues is not sufficient to gain ligand dissociation ability. Although the receptor had not lost the capacity to bind ligand, it was retained intracellularly after endocytosis either with or without bound ligand.

To investigate the fate of ligand dissociation deficient LDLR mutants, we extended the research described above with the construction of a reciprocal hybrid receptor ($LDLR_{1-292}LpR_{343-850}$) and two LDLR mutants, the His562 of which were replaced by Tyr and Asn, respectively. In contrast to LDLRΔEGF, all mutant receptors appeared to bind and internalize LDL when expressed in CHO cells, and the ligand remains visible in intracellular vesicles for at least 1 h, as assessed with fluorescence microscopy. Colocalization with internalized transferrin (Tf), a ligand that is known to efficiently recycle via the ERC upon Tf receptor (TfR)-mediated endocytosis (Yamashiro et al., 1984), shows that the lipoprotein receptors are present in the ERC in the absence of ligand, suggesting that constitutive recycling is not impaired. However, when LDL was added for a prolonged period, constitutive receptor recycling dramatically decreased, whereas only a minor reduction in recycling was observed for wt receptors, which implies that both mutant receptors as well as $LDLR_{1-292}LpR_{343-850}$ have lost the ability to recycle after ligand endocytosis. In addition, it implicates that these mutations affect only ligand dissociation rather than recycling of the receptors, like LDLRΔEGF (J.L. Goldstein, 2003, personal communication). From these observations, we propose that, although it is unanimously accepted that FH class 5 LDLR mutations lead to elevated plasma cholesterol levels (for reviews see Hobbs et al., 1990, 1992), not all of these mutations affect recycling of LDLR in the absence of LDL. Some specifically impair the dissociation step, whereas constitutive recycling of LDLR is maintained.

Materials and methods

Antibodies, reagents and proteins

Polyclonal anti-LpR 2189/90 rabbit antibody was raised against a synthetic peptide representing the unique N-terminal 20 amino acids (34-53) of the first cysteine-rich

repeat of LpR (Van Hoof et al., 2003); anti-LDLR C7 mouse antibody and anti-LDLR 121 rabbit antibody (Anderson et al., 1982) were generous gifts from Drs. Ineke Braakman and Jürgen Gent (Department of Bio-Organic Chemistry, Utrecht University, Utrecht, The Netherlands). Geneticin (G-418) (GibcoBRL), Zeocin (Invivogen), precision protein standards prestained broad range marker (Bio-Rad), alkaline phosphatase-conjugated affinipure goat-anti-rabbit IgG (AP-GAR), TRITC-conjugated goat-anti-rabbit IgG (TRITC-GAR) and TRITC-conjugated goat-anti-mouse IgG (TRITC-GAM) (Jackson ImmunoResearch Laboratories Inc.), leupeptin, aprotinin (Sigma), Oregon Green 488 (OG) carboxylic acid, and OG-labeled Tf (OG-Tf) (Molecular Probes), BSA and cold water fish gelatin (Sigma) were obtained from commercial sources. Human LDL was isolated from blood plasma (Bloedbank Midden Nederland) as described by Redgrave et al. (1975); Lp was isolated from locust hemolymph by ultracentrifugation as described by Van Hoof et al. (2002).

Cell culture

Wild-type Chinese hamster ovary (CHO) cells and CHO cells that do not express functional LDLR (*ldlA*; Kingsley and Krieger, 1984) were cultured in 25 cm^2 polystyrene culture flasks (CellStar) with growth medium containing HAM's F-10 Nutrient mixture, 10% heat inactivated fetal bovine serum, 100 U/ml penicillin G sodium and 100 µg/ml streptomycin sulfate in 85% saline (Gibco BRL) at 37°C in a humidified atmosphere of 5% CO_2. Growth medium of cells transfected with pcDNA3 or pcDNA3.1/Zeo(+) plasmid was supplemented with 300 µg/ml G-418, or 400 µg/ml Zeocin, respectively. In addition, 0.2 µM mevalonate was added to growth medium of *ldlA*(LDLR) and *ldlA*(LDLRΔEGF) transfectants (Davis et al., 1987; a generous gift from Dr. Joseph Goldstein, Department of Molecular Genetics, University of Texas Southwestern Medical Center at Dallas, Texas, USA). For fluorescence microscopy, cells were grown on glass cover slips (∅ 15 mm; Menzel-Gläser) in 12-wells (3.5 cm^2/well) multidishes (Costar), respectively.

Construction of mammalian expression vectors harboring $LDLR_{H562Y}$, $LDLR_{H562N}$, and $LDLR_{1-292}LpR_{343-850}$ cDNA constructs

pcDNA3-$LDLR_{H562Y}$ and pcDNA3-$LDLR_{H562N}$, were generated with QuickChange PCR using PfuTurbo DNA polymerase (Stratagene) according to manufacturer's protocol. The DNA mutations C1747T (amino acid mutation H562Y) and C1747A (amino acid mutation H562N) were generated with QuickChange PCR using primer 5'-GGGTTGAC TCCAAACTTTACTCCATCTCAAGCATCG in combination with 5'-CGATGCTTGA GATGGAGTAAAGTTTGGAGTCAACCC, and primer 5'-GGGTTGACTCCAAACT TAACTCCATCTCAAGCATCG in combination with 5'-CGATGCTTGAGATGGAGT TAAGTTTGGAGTCAACCC, respectively, and pBS-LDLR (a generous gift from Dr. Ineke Braakman, Department of Bio-Organic Chemistry, Utrecht University, Utrecht, The Netherlands) as template DNA. The constructs were subcloned into pcDNA3.1/Zeo(+) (Invitrogen) using the *Kpn*I and *Not*I (New England BioLabs) restriction enzymes screened for clones with the specific mutations by sequencing.

pcDNA3-$LDLR_{1-292}LpR_{343-850}$ was constructed with two PCR fragments, encoding the ligand binding domain of LDLR, and the region from EGF domain to intracellular C-terminus of LpR. The LDLR ligand binding domain encoding sequence was generated with PCR using primer 5'-AAGCTTCTGGCAGAGGCTGCGAGC (introduced *Hin*dIII site) in combination with 5'-GGTACCGCACTCTTTGATGGG

(introduced *Kpn*I site), and the pBS-LDLR plasmid as template DNA. The PCR fragment was ligated into pGEM-T (Promega), and the resulting plasmid (pGEM-T-$LDLR_{1-292}$) was screened for clones without mutations by sequencing. Similarly, the region encoding from EGF domain to intracellular tail of LpR was generated with PCR using primer 5'-GGTACCAATGAATGTGCTGTAAATAATGG (introduced *Kpn*I site) in combination with 5'-GCGGCCGCTTATACATAATCATTTGTCCC (introduced *Not*I site) with piLR-e (Dantuma et al., 1999) as template DNA. The PCR fragment was ligated into pGEM-T, the resulting plasmid (pGEM-T-$LpR_{343-850}$) was screened for clones without mutations by sequencing. The PCR fragment in pGEM-T-$LpR_{343-850}$ was ligated into pGEM-T-$LDLR_{1-292}$ after digestion with *Kpn*I and *Not*I (New England BioLabs), latter restriction site of which resides in the multiple cloning site of pGEM-T. Before transformation, the plasmid was redigested with *Spe*I to linearize pGEM-T-$LDLR_{1-292}$ without the $LpR_{343-850}$ insert. The resulting intermediary construct (pGEM-T-$LDLR_{1-292}LpR_{343-850}$) was digested with *Kpn*I and *Not*I (New England BioLabs), and ligated into pcDNA3.1/Zeo(+) that was treated with the same restriction enzymes to generate pcDNA3-$LDLR_{1-292}LpR_{343-850}$.

Generation of CHO cell lines stably expressing $LDLR_{H562Y}$, $LDLR_{H562N}$, and $LDLR_{1-292}LpR_{343-850}$

ldlA cells (Kingsley and Krieger, 1984) were grown to ~90% confluency 12-wells multidishes (Costar) and transfected for 16 h with 3 μg of pcDNA3-$LDLR_{H562Y}$, pcDNA3-$LDLR_{H562N}$ or pcDNA3-$LDLR_{1-292}LpR_{343-850}$ plasmid DNA. Transfection was conducted in 1 ml growth medium supplemented with 5 μl Lipofectamine2000 reagent (Invitrogen Life Technologies) according to the supplier's protocol with. After transfection, cells were transferred to 75 cm^2 culture flasks (CellStar), and grown for 10 days in selective growth medium, containing 400 μg/ml Zeocin to obtain transfectants stably expressing the hybrid receptors.

Western blot analysis of cell membrane extracts

CHO cells were harvested from 25 cm^2 culture flasks (CellStar) at ~90% confluency and resuspended in CHAPS buffer (20 mM HEPES, 124 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 2.5 mM Na_2HPO_4, 1.2 mM $MgSO_4$, 1 mM EDTA, 0.1 mM benzamidine, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1% CHAPS), incubated for 10 min on ice, and spun down at 15000 *g* for 10 min at 4°C. Supernatants were dissolved in Laemmli buffer (Laemmli, 1970) without β-mercaptoethanol and 0.025% SDS, prior to separation by SDS-PAGE on a 10% polyacrylamide gel as described by Van Hoof et al. (2002). The separated membrane proteins were transferred to polyvinylidene fluoride membrane (Millipore) by Western blotting and incubated with anti-LDLR C7 mouse antibody (1:2000), or anti-iLR 2189/90 rabbit antibody (1:200) for 2 h, followed by 1 h AP-GAM or AP-GAR incubation. Hybridized secondary antibody was visualized by incubating the blot in TSM buffer, containing 100 mM Tris-HCl, 100 mM NaCl, 10 mM $MgAc_2$, 50 μg/ml p-nitro blue tetrazolium chloride (NBT; Boehringer Mannheim), 25 μg/ml 5-bromo-4-chloro-3-indoyl-phosphate p-toluidine (BCIP; Roche Diagnostics), pH 9.0.

Incubation of cells with fluorescently-labeled ligands

Human LDL (1 mg/ml) and insect Lp (1 mg/ml) were fluorescently labeled with 20 μl/ml OG dissolved in DMSO (1 μg/μl) at room temperature under continuous

stirring for 1 h according to the manufacturer's instructions. Fluorescently-labeled lipoprotein was purified with Sephadex G-25 PD-10 columns (Amersham Pharmacia Biotech) to replace the PBS by incubation medium containing 10 mM HEPES, 50 mM NaCl, 10 mM KCl, 5 mM $CaCl_2$, 2 mM $MgSO_4$, pH 7.4. For endocytic uptake, cells were seeded in 12-well multidishes at 20% confluency, and cultured for 2 days in a humidified atmosphere at 37°C. Thereafter, cells were incubated for 15 min at 37°C with 35 μg/ml OG-LDL, 25 μg/ml OG-Lp, or 25 μg/ml OG-Tf, 25 μg/ml as indicated. After incubatin, cells were rinsed in incubation medium and either directly fixated in 4% paraformaldehyde diluted in PBS for 30 min at room temperature, or first chased in growth medium at 37°C as indicated.

Immunofluorescence

Immunofluorescence (IF) was used to visualize receptors overexpressed in the transfectants. Fixated cells were washed twice with PBS buffer and permeabilized with PBS buffer supplemented with 0.4% Triton X-100 (PBSX) for 5 min at room temperature. The cells were subsequently incubated with PBSX containing 50 mM glycin for 10 min and 5% BSA for 30 min at room temperature. The cells were blocked for 5 min with 0.1% cold water fish gelatin in PBSX (PBSXG) at room temperature and incubated with corresponding primary antibodies (1:500) for 1 h at 37°C. After rinsing 4 times for 5 min with PBSX and a final wash-step of 5 min in PBSXG at room temperature, the samples were processed for indirect immunofluorescence by incubation with TRITC-labeled secondary antibody for 30 min at 37°C and rinsed an additional 4 times with PBSXG.

Fluorescence microscopy and image processing

Cover slips with fixated cells were mounted in Mowiol and examined on a fluorescence Axioscop microscope (Zeiss) with a Hg HBO-50 lamp and a Plan-Neofluar 100×/1.30 oil lens. Using FITC/TRITC filters, digital images were acquired with a DXM 1200 digital camera and ACT-1 version 2.00 software (Nikon Corporation). Images were processed using Scion Image beta version 4.0.2 (Scion Corporation) and PaintShop pro 7.00 (Jasc Software) software.

Quantification of receptor recycling efficiency was conducted by incubating the cells with OG-LDL or OG-Lp as described, after which the receptor was visualized with IF. The amount of cells in which the receptors had perceptibly converged in the ERC was divided by the total number of cells that were capable of internalizing OG-labeled ligand. Each data set was the result of a duplo experiment, except when indicated.

Results

Expression of $LDLR_{H562Y}$, $LDLR_{H562N}$, and $LDLR_{1-292}LpR_{343-850}$ by stably transfected CHO cells

To analyze the effect of His562 mutation on dissociation of LDL and recycling of the receptor, two LDLR mutants were created, His562 of which was substituted by Tyr and Asn, respectively ($LDLR_{H562Y}$ and $LDLR_{H562N}$; Fig. 1A). In addition, an LDLR-LpR hybrid receptor was constructed to assess the essentiality of the LDLR EGF domain in LDL binding, uncoupling, and receptor recycling. To this end, the ligand binding

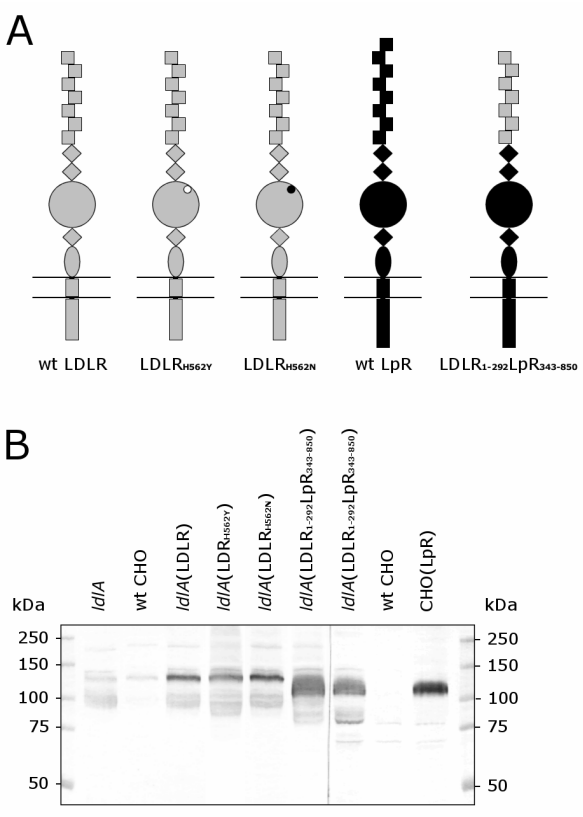

Figure 1. Schematic models of wt LDLR, LDLR$_{H562Y}$, LDLR$_{H562N}$, wt LpR, and LDLR$_{1-292}$LpR$_{343-850}$; LDLR domains are depicted in grey, and LpR domains in black (A). LDLR is composed of a ligand binding domain harboring 7 cysteine-rich repeats (squares), an EGF domain comprising two consecutive EGF repeats (diamonds) that are separated from a third by a β-propeller (circle), an O-linked glycosylation domain (oval), a transmembrane domain (short rectangle), and an intracellular tail (long rectangle). His562 of the mutants is substituted by Tyr or Asn. The domain arrangement of LpR is identical to LDLR; however, the ligand binding cluster of LpR contains 8 cysteine-rich repeats, and the intracellular tail is longer. LDLR$_{1-292}$LpR$_{343-850}$ harbors the ligand binding domain of LDLR, whereas its region from EGF domain to intracellular C-terminus is derived from LpR. (B) Expression of receptors in transfected CHO cell lines was analyzed on Western blot. Membrane proteins were isolated from LDLR-deficient *ldlA* cells (lane 1; Kingsley and Krieger, 1984), wt CHO cells (lanes 2 and 8), polyclonal cell lines *ldlA*(LDLR) (lane 3), *ldlA*(LDLR$_{H562Y}$) (lane 4), *ldlA*(LDLR$_{H562N}$) (lane 5), and *ldlA*(LDLR$_{1-292}$LpR$_{343-850}$) (lanes 6 and 7), and the monoclonal cell line CHO(LpR) (lane 9; Van Hoof et al., 2002) as described in the materials and methods section. Samples were dissolved in Laemmli buffer (Laemmli, 1970) containing 0.025% SDS and no reducing agents, and directly subjected to SDS-PAGE under non-reducing conditions. Following transfer to polyvinylidene fluoride membrane, lanes 1-6 were incubated with anti-LDLR 121 rabbit antibody, and lanes 7-9 with anti-LpR 2189/90 rabbit antibody. The molecular weight markers (kDa) are indicated on the very left and right of the blot.

domain of *L. migratoria* LpR (Fig. 1A) was replaced by that of human LDLR (LDLR$_{1-292}$LpR$_{343-850}$; Fig. 1A). LDLR-deficient CHO cells (*ldlA* cells; Kingsley and Krieger, 1984) were stably transfected with the mammalian expression vector pcDNA3 harboring wt LDLR, LDLR$_{H562Y}$, LDLR$_{H562N}$, or LDLR$_{1-292}$LpR$_{343-850}$ recombinant DNA construct, generating polyclonal *ldlA*(LDLR), *ldlA*(LDLR$_{H562Y}$), *ldlA*(LDLR$_{H562N}$), and *ldlA*(LDLR$_{1-292}$LpR$_{343-850}$) cell lines, respectively. The receptors expressed by these transfectants were analyzed and compared, using detergent cell extracts that were separated by SDS-PAGE under non-reducing conditions. The proteins were transferred to polyvinylidene fluoride membrane and immunoblotted with polyclonal anti-LDLR 121 rabbit antibody specifically recognizing non-reduced LDLR (Fig. 1B, lanes 1-6; Van Hoof et al., 2002), and anti-LpR 9218 rabbit antibody (Fig. 1B, lanes 7-9) raised against the 19 C-terminal amino acid residues of LpR (Van Hoof et al., 2002). Western blotting shows that under these conditions, the electrophoretic mobility of both His562 mutants (Fig. 1B, lanes 4 and 5) is identical to endogenous CHO LDLR (Fig. 1B,

lane 2) and wt LDLR (Fig. 1B, lane 3), whereas that of $LDLR_{1-292}LpR_{343-850}$ is higher (Fig. 1B, lane 6). $LDLR_{1-292}LpR_{343-850}$ harbors the O-linked glycosylation domain of LpR that is not as heavily glycosylated as the LDLR O-linked glycosylation domain (Davis et al., 1986, Van Hoof et al., 2002), and has an electrophoretic mobility similar to wt LpR (compare Fig. 1B, lanes 7 and 9). The apparent molecular weight of wt LDLR and wt LpR under non-reducing conditions (Fig. 1B) is higher than under reducing conditions as shown earlier (Davis et al., 1986; Van Hoof et al., 2002). Decrease in electrophoretic mobility under latter conditions coincides with reduction of multiple disulfide bonds present in the ligand binding domain and EGF domain of lipoprotein receptors. The mobility of both LDLR mutants and $LDLR_{1-292}LpR_{343-850}$ under non-reducing conditions is similar to that of wt LDLR and wt LpR, respectively, which strongy suggests that all receptors are folded correctly.

Endocytosis and intracellular trafficking of LDL and receptor in *ldlA*($LDLR_{H562Y}$), *ldlA*($LDLR_{H562N}$), and *ldlA*($LDLR_{1-292}LpR_{343-850}$) transfectants

To test *ldlA*($LDLR_{H562Y}$), *ldlA*($LDLR_{H562N}$), and *ldlA*($LDLR_{1-292}LpR_{343-850}$) transfectants for functional expression of receptor, the cells were incubated for 15 min at 37°C in a HEPES-supplemented buffer (incubation medium) that contained Oregon Green 488 (OG)-labeled human LDL (OG-LDL), and compared with *ldlA*(LDLR) transfectants. Fluorescence microscopy revealed that all LDLR variants mediate endocytic uptake of OG-LDL. In contrast to non-transfected *ldlA* cells (data not shown), *ldlA*(LDLR) transfectants (Fig. 2A) show a punctate fluorescence pattern representing endosomes that contain OG-LDL. In *ldlA*($LDLR_{H562Y}$), *ldlA*($LDLR_{H562N}$), and *ldlA*($LDLR_{1-292}LpR_{343-850}$) transfectants, a similar staining pattern was observed (Fig. 2B-D). In addition to wt LDLR, $LDLR_{H562Y}$, $LDLR_{H562N}$, and $LDLR_{1-292}LpR_{343-850}$, anti-LDLR 121 polyclonal antibody recognizes non-functional intracellular LDLR-intermediates expressed by *ldlA* cells under native, non-reducing conditions (Fig. 1B, lane 1; Van Hoof et al., 2002). The use of anti-LDLR C7 mouse antibody, which specifically binds to the properly-folded first cysteine-rich repeat of the LDLR ligand binding domain (Anderson et al., 1982), allows exclusive visualization of the receptors overexpressed in the transfectants. LDLR recycles continuously without antecedent ligand binding (for review see Goldstein et al., 1979), and is prominently present in the ERC. Tf converges in the ERC after receptor-mediated endocytosis by endogenously expressed TfR (Yamashiro et al., 1984; McGraw et al., 1987; Van Hoof et al., 2002). Incubation of *ldlA*(LDLR) cells with OG-labeled Tf for 15 min at 37°C, followed by a chase for 10 min in growth medium without ligand shows that Tf (Fig. 2E) colocalizes with wt LDLR (Fig. 2F) in the ERC (Fig. 2G), which indicates that LDLR overexpressed in *ldlA* cells recycles normally. Under similar conditions, the receptors in *ldlA*($LDLR_{H562Y}$), *ldlA*($LDLR_{H562N}$), and *ldlA*($LDLR_{1-292}LpR_{343-850}$) transfectants also prominently converged in the ERC as confirmed by colocalization with Tf (Fig.2H-P), suggesting that these receptors also recycle constitutively.

A chase of 60 min at 37°C in growth medium without ligand after a 15 min pulse with OG-LDL did neither affect the intracellular vesicle staining pattern, nor significantly reduce the fluorescence intensity in *ldlA*(LDLR) transfectants (Fig. 3A); the receptor was predominantly localized in the ERC (Fig. 3B) and did not colocalize with LDL (Fig. 3C). This indicates that the ligand is most likely delivered to lysosomes for degradation, whereas the receptor continuously recycles. Similar to *ldlA*(LDLR),

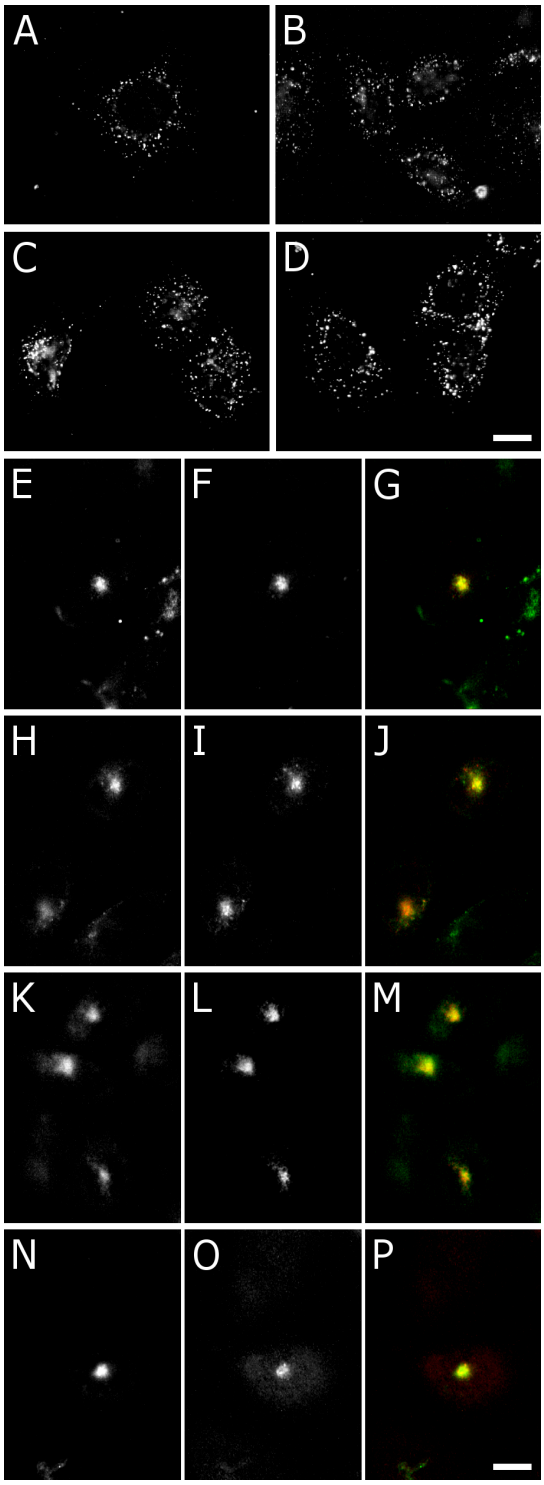

Figure 2. Receptor-mediated endocytic uptake of fluorescently-labeled LDL by *ldlA*(LDLR) (A), *ldlA*($LDLR_{H562Y}$) (B), *ldlA*($LDLR_{H562N}$) (C), and *ldlA*($LDLR_{1-292}LpR_{343-850}$) (D) transfectants. (A-D) Cells were incubated with OG-LDL in incubation medium for 15 min at 37°C, fixated with paraformaldehyde, and mounted in mowiol. (E-G) Incubation of *ldlA*(LDLR) transfectants for 15 min with OG-Tf, followed by a short chase of 10 min in growth medium shows that Tf accumulates in the ERC (E). IF using anti-LDLR C7 mouse antibody reveals that wt LDLR (F), is also localized in this organelle (G). Under identical conditions, $LDLR_{H562Y}$, $LDLR_{H562N}$, and $LDLR_{1-292}LpR_{343-850}$ also colocalize with Tf in the ERC (H-P). LDL is shown in grey (A-D); Tf is shown in grey (E, H, K, and N) or green (G, J, M, and P); receptor is shown in grey (F, I, L, and O) or red (G, J, M, and P); colocalization is shown in yellow (G, J, M, and P). Bars, 10 μm (D and P).

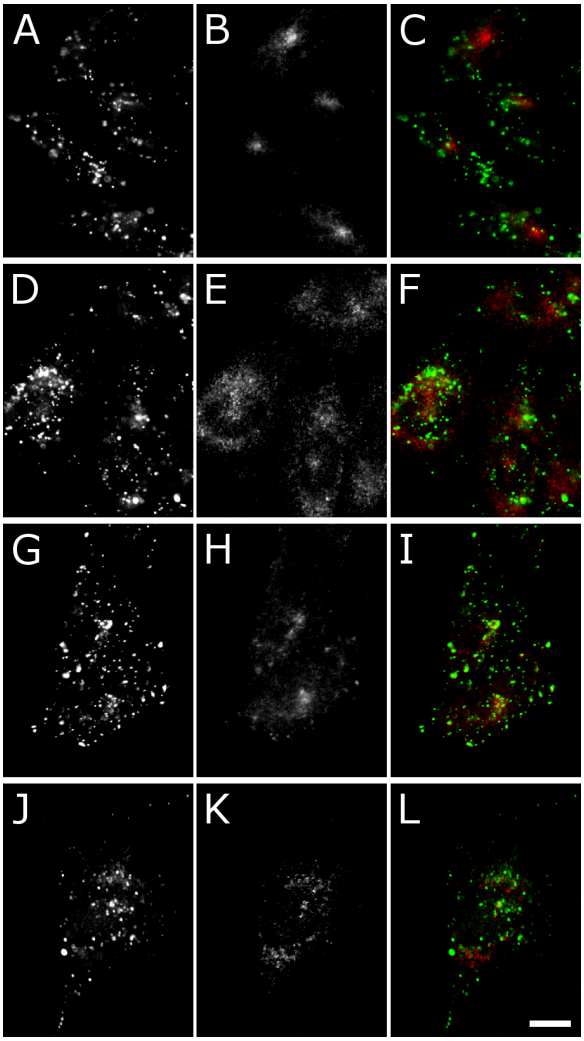

Figure 3. Internalized LDL is retained in intracellular vesicles after 60 min upon endocytosis by wt LDLR, as well as $LDLR_{H562Y}$, $LDLR_{H562N}$, and $LDLR_{1-292}LpR_{343-850}$. (A-L) Transfectants were preincubated for 15 min with OG-LDL, followed by a 60 min chase in normal growth medium without ligand; the receptor was stained with IF using anti-LDLR C7 mouse antibody. Analysis with fluorescence microscopy shows that LDL (A) resides in intracellular vesicles (i.e. lysosomes) after endocytic uptake by wt LDLR. In contrast, the receptor recycles and is predominantly present in the ERC (B); ligand and receptor do not colocalize (C). In *ldlA*($LDLR_{H562Y}$) transfectants, LDL is also present in vesicles after a similar pulse and chase (D); however, the receptor is not highly concentrated in the ERC (E), which reduces its visibility with fluorescence microscopy considerably. This renders it difficult to determine if receptor and ligand colocalize (F). Identical results were obtained when *ldlA*($LDLR_{H562N}$) and *ldlA*($LDLR_{1-292}LpR_{343-850}$) transfectants were subjected to the same assay (G-I and J-K, respectively). LDL is shown in grey (A, D, G, and J) or green (C, F, I, and L); receptor is shown in grey (B, E, H, and K) or red (C, F, I, and L); colocalization is shown in yellow (C, F, I, and L). Bar, 10 μm (L).

LDL remained in vesicular compartments in *ldlA*($LDLR_{H562Y}$), *ldlA*($LDLR_{H562N}$), and *ldlA*($LDLR_{1-292}LpR_{343-850}$) transfectants; however, the amount of ERC-located receptor had decreased to some extent (Fig. 3D-L) compared to that in the absence of LDL (Fig. 2H-P). These data suggest that, even though LDL is transported to lysosomes in all transfectants, recycling of $LDLR_{H562Y}$, $LDLR_{H562N}$, and $LDLR_{1-292}LpR_{343-850}$ appears to be affected.

Reduction of receptor recycling efficiency in *ldlA*($LDLR_{H562Y}$), *ldlA*($LDLR_{H562N}$), and *ldlA*($LDLR_{1-292}LpR_{343-850}$) transfectants upon prolonged LDL incubation

Whereas mutation of His562 to Tyr is presumed to result in impaired LDLR recycling *in vivo* (Sun et al., 1994; J.C. Defesche, 2003, personal communication), the *in vitro*

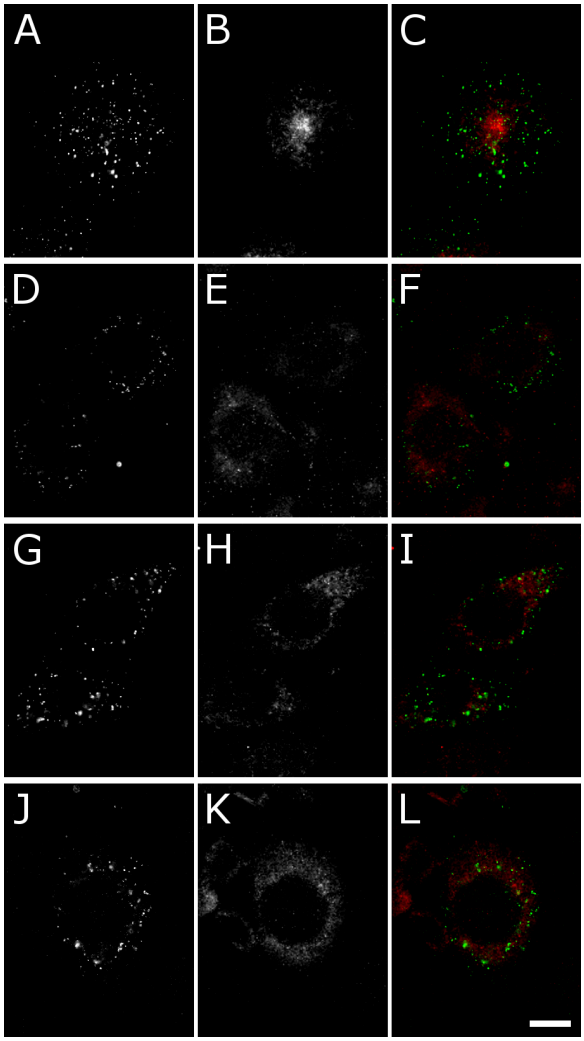

Figure 4. In contrast to *ldlA*(LDLR) transfectants, LDL uptake by *ldlA*($LDLR_{H562Y}$), *ldlA*($LDLR_{H562N}$), and *ldlA*($LDLR_{1-292}LpR_{343-850}$) transfectants is reduced, and receptors are not visibly localized in the ERC after prolonged incubation with LDL. (A-L) Transfectants were preincubated for 90 min in growth medium with unlabeled LDL, followed by a 15 min pulse with OG-LDL; the receptor was stained with IF using anti-LDLR C7 mouse antibody. Fluorescence microscopy reveals that LDL uptake (A) by wt LDLR (B) is not visibly affected by prolonged preincubation, and ligand and receptor do not colocalize (C). Under similar conditions, endocytosis of LDL (D) by *ldlA*($LDLR_{H562Y}$) transfectants is decreased, and the receptors have not converged in the ERC (E), which renders colocalization analysis impossible (F). Under similar conditions, identical results are obtained with *ldlA*($LDLR_{H562N}$) and *ldlA*($LDLR_{1-292}LpR_{343-850}$) transfectants (G-I and J-K, respectively). LDL is shown in grey (A, D, G, and J) or green (C, F, I, and L); receptor is shown in grey (B, E, H, and K) or red (C, F, I, and L); colocalization is shown in yellow (C, F, I, and L). Bar, 10 μm (L).

experiments described above show that this LDLR mutant as well as $LDLR_{H562N}$ and the $LDLR_{1-292}LpR_{343-850}$ hybrid receptor are prominently present in the ERC in the absence of LDL. To mimic *in vivo* conditions in which LDL is continuously present, transfectants were preincubated for 90 min at 37°C in growth medium with unlabeled LDL, prior to a pulse with OG-LDL. Endocytic uptake of OG-LDL by wt LDLR (Fig. 4A) was not perceptibly reduced in comparison to a pulse without preincubation of unlabeled LDL (Fig. 2A). In addition, LDLR was eminently concentrated in the ERC (Fig. 4B) and did not colocalize with LDL (Fig. 4C), showing that continuous LDL endocytosis affects neither receptor recycling nor uptake of LDL. In contrast to wt LDLR, a significant decrease in receptor-mediated endocytosis of LDL by $LDLR_{H562Y}$ was observed under identical conditions (Fig. 4D). Moreover, the ERC was almost depleted of $LDLR_{H562Y}$ (Fig. 4E and F); instead of converging in the ERC, the receptor appeared to be scattered throughout the cel interior, which makes it almost impossible to

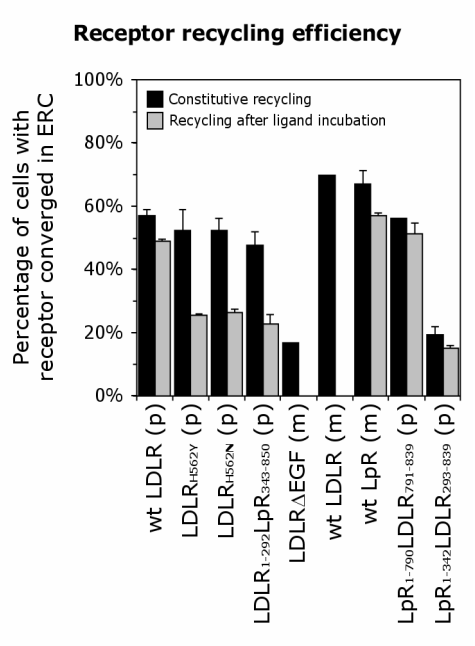

Figure 5. Constitutive RRE (black) and RRE after prolonged LDL incubation (grey) in transfected cell lines. Polyclonal cell lines are indicated with (p); monoclonal cell lines are indicated with (m); error bars show SD of a duplicate experiment, except for LDLRΔEGF (m) and wt LDLR (m) (single experiment); LpR$_{1-790}$LDLR$_{791-839}$ SD<0.1% (duplicate experiment).

discern with fluorescence microscopy. LDL endocytosis by LDLR$_{H562N}$ and LDLR$_{1-292}$LpR$_{343-850}$, as well as intracellular distribution of these receptors was similarly affected by extensive preincubation with LDL (Fig. 4G-L), suggesting that recycling of both mutant receptors and the hybrid receptor is hindered in the presence of LDL.

Quantification of receptor recycling efficiency in *ldlA*(LDLR$_{H562Y}$), *ldlA*(LDLR$_{H562N}$), and *ldlA*(LDLR$_{1-292}$LpR$_{343-850}$) transfectants after prolonged LDL incubation

To quantify receptor recycling efficiency (RRE) upon incubation with LDL, the relative number of transfectants in which the receptors perceptibly converged in the ERC was determined after a pulse with fluorescently-labeled LDL. The cells were incubated with OG-LDL for 15 min at 37°C; the receptor was visualized with anti-LDLR C7 mouse antibody. Analysis with fluorescence microscopy revealed that in approximately 57% of polyclonal *ldlA*(LDLR) transfectants that had internalized OG-LDL, wt LDLR was prominently localized in the ERC (Fig. 5). The HEPES component of incubation medium inhibits acidification of the endosome lumen (Sullivan et al., 1987), thus prevents LDL release after receptor-mediated endocytosis, and transition of LDLR to the ERC. Under these conditions, ERC-located LDLR represents the pool of receptors that were internalized prior to LDL incubation, and in the process of recycling. The percentage of cells in which LDLR was discernable situated in the ERC is an indication for constitutive RRE of wt LDLR in a polyclonal transfected cell line. The constitutive RRE of LDLR$_{H562Y}$, LDLR$_{H562N}$, and LDLR$_{1-292}$LpR$_{343-850}$ after an identical pulse closely matched that of wt LDLR (Fig. 5), which suggests that these receptors are not affected in their ability to constantly recycle. In contrast, constitutive RRE of LDLRΔEGF in monoclonal *ldlA*(LDLRΔEGF) transfectants was found to be 3-fold lower (Fig. 5), which indicates that this receptor is not efficiently transferred to the ERC

after internalization (J.L. Goldstein, 2003, personal communication). Because this decrease was not observed for the LDL-binding mutants, these findings support that $LDLR_{H562Y}$, $LDLR_{H562N}$, and $LDLR_{1-292}LpR_{343-850}$ are able to efficiently recycle when no ligand is present.

Constitutive RRE of wt LDLR in monoclonal *ldlA*(LDLR) transfectants (Fig. 5) is slightly higher than in a polyclonal cell line (Fig. 5); however, similar to wt LpR in a monoclonal cell line (Fig. 5; Van Hoof et al., 2002). Conversely, a polyclonal cell line expressing the $LpR_{1-790}LDLR_{791-839}$ hybrid receptor (Chapter 5), has a constitutive RRE almost identical to wt LDLR in polyclonal *ldlA*(LDLR) cells; however, lower than monoclonal LpR-tranfected cells (Fig. 5), even though this receptor was observed to recycle normally. Thus, reduced constitutive RRE in polyclonal cell lines is most likely the result of variability among individual transfectants. In contrast, the decrease in constitutive RRE of $LpR_{1-342}LDLR_{293-839}$ (Fig. 5; Chapter 5) was found to be similar to LDLRΔEGF (Fig. 5; J.L. Goldstein, 2003, personal communication), which indicates a significant reduction of RRE due to the mutation.

As described above, recycling of ligand-free receptors is most likely not impaired when His562 is substituted by Tyr or Asn, or the complete region from EGF domain to intracellular tail is replaced by that of LpR ($LDLR_{1-292}LpR_{343-850}$). However, a decrease in ERC-located receptors upon prolonged LDL incubation of *ldlA* cells transfected with these receptors (Fig. 4) suggests that the ligand inhibits efficient recycling via the ERC. To quantify the effect of LDL on RRE when LDL is continuously present, *ldlA*(LDLR), *ldlA*($LDLR_{H562Y}$), *ldlA*($LDLR_{H562N}$), and *ldlA*($LDLR_{1-292}LpR_{343-850}$) transfectants were preincubated with unlabeled LDL for 90 min at 37°C in normal growth medium, followed by a 15 min pulse with OG-LDL. After incubation, the receptors were labeled with anti-LDLR C7 mouse antibody and visualized with fluorescence microscopy as described above. Whereas under these conditions RRE of wt LDLR in the polyclonal line decreased less than 10% (Fig. 5), the mutants $LDLR_{H562Y}$, $LDLR_{H562N}$, and hybrid receptor $LDLR_{1-292}LpR_{343-850}$ showed more than 2-fold decrease in RRE (Fig. 5). The efficiency of these mutated receptors to recycle in the continuous presence of LDL is similar to that of LDLRΔEGF in the absence of ligand. These findings strongly suggest that, in contrast to LDLRΔEGF, mutation of His562 to Tyr or Asn does not affect ligand-free receptor recycling, but most likely inhibits ligand dissociation, upon which access of the receptor to the ERC is denied. The same assay was used to determine the effect of prolonged Lp incubation on wt LpR, $LpR_{1-790}LDLR_{791-839}$, and $LpR_{1-342}LDLR_{293-839}$; however, did not result in significant alteration of RRE (Fig. 5). When in complex with Lp, wt LpR as well as $LpR_{1-790}LDLR_{791-839}$ continued to recycle, whereas $LpR_{1-342}LDLR_{293-839}$ hybrid receptors did not significantly converge in the ERC. Taken together, our *in vitro* experiments indicate that *in vivo* some FH class 5 mutations result in impairment of ligand dissociation (H562Y; Sun et al., 1994; J.C. Defesche, 2003, personal communication), whereas others inhibit constitutive recycling (LDLRΔEGF; Miyake et al., 1989).

Discussion

Numerous naturally occurring mutations in the LDLR gene have been found to result in elevated plasma cholesterol levels *in vivo*. These mutations have been classified

according to their effect on the intricate process of plasma LDL removal by LDLR (for review see Hobbs et al., 1990, 1992). Class 5 mutations impair receptor recycling, most of which have been mapped to the EGF domain, e.g. H562Y (Sun et al., 1994; J.C. Defesche, 2003, personal communication). Deletion of the complete EGF domain generates a receptor that maintains VLDL binding capacity. However, the property to bind LDL is lost (Davis et al., 1987), and ligand (i.e. VLDL) uncoupling is impaired. As a result, the receptor-ligand complex is degraded in lysosomes. In contrast to this LDLRΔEGF, deletion of the EGF domain of VLDLR does not result in receptor-ligand complex degradation. Instead, this VLDLRΔEGF was shown to recycle bound ligand, for which receptor-associated protein (RAP) was used, via the ERC (Mikhailenko et al., 1999). Moreover, the insect LDLR homologue, wt LpR containing a complete EGF domain, was shown to recycle its ligands Lp and RAP *in vitro* (Van Hoof et al., 2002). Apparently, highly homologous receptors follow different intracellular routes, which can be influenced by mutations that do not per definition result in a similar alteration of the receptor pathway. To analyze the effects of mutations that impair receptor recycling at the cellular level, we constructed two LDLR mutants in addition to a hybrid receptor.

The naturally occurring $LDLR_{H562Y}$ mutant leads to FH (Sun et al., 1994; J.C. Defesche, 2003, personal communication). To investigate the role of His562 in receptor recycling and the effect of the H562Y FH mutation on the intracellular receptor pathway, we transfected *ldlA* cells that do not express functional LDLR (Kingsley and Krieger, 1984) with $LDLR_{H562Y}$. Additionally, His562 was replaced by Asn, which corresponds to the amino acid residue in LpR as assessed with three-dimensional structure analysis. Because LpR recycles in complex with ligand when expressed *in vitro* (Van Hoof et al., 2002), it was expected that $LDLR_{H562N}$ also recycles bound ligand. Both receptors were observed to recycle through the ERC constitutively (Fig. 2H-M), and are able to internalize LDL (Fig. 2B and C). This indicates that these receptors are functionally expressed, and that His562 is not essential for LDL binding. In addition, internalized ligand was retained in the cell (Fig. 3D and G), demonstrating that H562N does not induce ligand recycling. Whereas the mutants recycle without antecedent ligand binding, RRE was significantly reduced upon prolonged LDL incubation (Fig. 4D-I and 5). The observation that receptor recycling is only impaired after ligand internalization strongly suggests that His562 is essential for ligand uncoupling; however, not for receptor recycling. Thus, the FH class 5 mutation H562Y (Sun et al., 1994; J.C. Defesche, 2003, personal communication) most likely impairs ligand dissociation rather than recycling of the receptor *in vivo*.

The absence of ligand recycling ability after substitution of His562 by Asn may be due to other necessary amino acid residues that are not present in LDLR. However, the hybrid receptor $LDLR_{1-292}LpR_{343-850}$, the ligand binding domain of which was derived from LDLR and the other domains from LpR, showed endocytic and recycling properties in *ldlA* transfectants that were similar to those observed for $LDLR_{H562Y}$ and $LDLR_{H562N}$. This suggests that the LpR ligand binding domain contains elements implicated in recycling of the LpR-ligand complex *in vitro*. However, when the ligand binding domain of LDLR was replaced by that of LpR, the resulting hybrid receptor ($LpR_{1-342}LDLR_{293-839}$) recycled neither constitutively, nor in complex with ligand (Fig. 5). The crystal structure of the LDLR ectodomain elucidated at low pH suggests that LDLR in endosomes adopts a conformation in which the ligand binding domain is folded onto the EGF domain (Rudenko et al., 2002). Therefore, it is very plausible that

the EGF domain has to recognize the ligand binding domain (i.e. establish intramolecular connections) in order to recycle. In that case, the ligand binding domain of LpR is most likely not recognized by the LDLR EGF domain. On the other hand, essential bonds can be formed between the ligand binding domain of LDLR and the LpR EGF domain in the absence of ligand. The resulting conformation enables the receptor (i.e. $LDLR_{1-292}LpR_{343-850}$) to escape lysosomal degradation.

Deletion of the LDLR EGF domain abolishes LDL binding by LDLRΔEGF, whereas VLDL binding is maintained, which implies that the EGF domain of LDLR is important for LDL binding (Davis et al., 1987). However, LDL endocytosis by $LDLR_{1-292}LpR_{343-850}$ was not impaired upon replacement of the LDLR EGF domain by that of LpR (Fig. 2D). This suggests that either the EGF domain of LpR can take over the LDL binding property of the LDLR EGF domain, or LDL binding is sterically hindered (e.g. by the O-linked glycosylation domain) when the EGF domain is removed. In latter case, the EGF domain would merely function as a spacer region for LDL binding that can be replaced by the EGF domain of LpR. Support for minor importance of LDLR EGF domain in LDL binding was obtained from studies with $LpR_{1-342}LDLR_{293-839}$. This hybrid receptor harbors the EGF domain of LDLR; however, is unable to bind LDL (D. Van Hoof, K.W. Rodenburg, D.J. Van der Horst, 2003, unpublished observations). This indicates that the LDLR EGF domain has not sufficient affinity for LDL to form a stable complex. VLDLRΔEGF was not tested for VLDL binding (D.K. Strickland, 2003, personal communication); alternatively, RAP, a chaperone protein (for review see Bu and Schwartz, 1998; Bu and Marzolo, 2000) that binds with high affinity to VLDLR (Battey et al., 1994), was used for analysis of the deletion mutant (Mikhailenko et al., 1999). Because LpR was shown to mediate endocytosis of Lp as well as RAP (Van Hoof et al., 2002), deletion of the LpR EGF domain will provide insight into the role of this domain during ligand binding by lipoprotein receptors. In contrast to LDLRΔEGF (Fig. 5; J.L. Goldstein, 2003, personal communication), VLDLRΔEGF was observed to recycle constitutively without antecedent ligand binding (D.K. Strickland, 2003, personal communication). However, it cannot be excluded that endogenously expressed ligands (e.g. RAP, and urokinase plasminogen activator-plasminogen activator inhibitor-1 complex) attach to the receptor and recycle as a complex (D.K. Strickland, 2003, personal communication). Irrespectively, the fate of endocytosed lipoprotein receptors with a disfunctional or missing EGF domain is receptor type specific. Analysis of LpRΔEGF will contribute to elucidating the essentiality of this domain in receptor-ligand complex recycling of lipoprotein receptors in general.

A total of three His residues have been found to participate in sustaining the arched conformation of the LDLR ectodomain at endosomal pH (Rudenko et al., 2002). His586 is positioned in close proximity to His562 in the EGF domain, and presumed to form a salt bridge with Asp149 located in the fourth ligand binding repeat of the ligand binding domain. Although FH mutations in which His586 is substituted have not been described as yet, mutational analysis *in vitro* may elucidate whether this residue is important for ligand release and receptor recycling. On the other hand, mutation of His190 to Tyr has been found to result in FH (Hopkins et al., 1999). His190 resides in the fifth ligand binding repeat of the ligand binding domain; however, it is not known whether LDL binding is impaired (P.N. Hopkins and M. Emi, 2004, personal communication). If this mutation does not inhibit binding of LDL, it may render $LDLR_{H190Y}$ incapable of uncoupling the ligand after endocytosis, resulting in degradation of the receptor-ligand

complex. Characterization of $LDLR_{H190Y}$ and additional FH mutations that are located in the ligand binding domain *in vitro* will disclose involvement of this domain in the ligand-dissociating step that precedes receptor recycling.

The findings with LDLR mutants and hybrid receptors suggest that recycling of LDLR after ligand endocytosis is a process that occurs in successive stages. In the initial step, bound ligand is released from the ligand binding domain of LDLR in the acidic lumen of the sorting endosome. Histidine residues in LDLR (e.g. His562, His190 and His586) become protonated in an acidic milieu, some of which are assumed to play a key role in the segregation step (Rudenko et al., 2002; for review see Innerarity, 2002; Jeon and Blacklow, 2003). If LDLR fails to uncouple LDL due to mutation or absence of essential residues, the receptor-ligand complex is destined to be degraded in lysosomes (Davis et al., 1987). Although studies with VLDLRΔEGF (Mikhailenko et al., 1999) as well as with wt LpR (Van Hoof et al., 2002) indicate that ligand dissociation is not a prerequisite for all lipoprotein receptors in order to recycle, it appears to be a crucial step for LDLR to maintain cholesterol homeostasis *in vivo* (for review see Hobbs et al., 1990, 1992). The second step most likely involves a conformational change of the LDLR ectodomain (Rudenko et al., 2002) in order to pass a quality-check that discriminates between receptors in a ligand free state, and those in complex with ligand. If LDL remains bound to LDLR, the ligand binding domain cannot fold onto the EGF domain and make contact with this domain (for review see Innerarity, 2002; Jeon and Blacklow, 2003). In addition, mutations in the EGF domain as well as the ligand binding domain could also inhibit formation or affect stability of the folded conformation. In either case, the receptor does not comply to the conditions necessary for recycling in the presence as well as absence of LDL. As a consequence, transfer to the cell surface is prohibited; the receptor is directed to lysosomes and degraded. Whereas some mutations impair only ligand dissociation (e.g. H562Y), others prevent conformational change (e.g. LDLRΔEGF). Taken together, the present observations imply that FH class 5 LDLR mutations can be subdivided into two distinct subclasses: those that inhibit ligand dissociation and those that impede recycling of the receptor.

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CHAPTER 7

VII

Summarizing discussion

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Lipids constitute a group of vital molecules in nature, displaying functions from building blocks (e.g. for the plasma membrane, which defines the barrier between the interior of a living cell and its environment) to high-energy sources for numerous biological processes. Specific lipid-protein complexes (lipoproteins) mediate the transport of lipids through the circulation of multicellular organisms. A large variety of lipoproteins with different functions and heterogenous compositions has been identified and characterized in mammals (Frayn, 1996; Ganong, 2001; Vance, 2002). Whereas some lipoproteins remain extracellularly and unload part of their lipid cargo at the cell surface (Bernlohr et al., 2002), other are taken up by the cell via receptor-mediated endocytosis (Fielding and Fielding, 2002). For example, internalization of low-density lipoprotein (LDL) is mediated by the LDL receptor (LDLR), after which the particle is completely degraded (for reviews see Goldstein et al., 1985; Brown and Goldstein, 1986).

In contrast to mammals, insects make use of a single multifunctional lipoprotein, lipophorin (Lp) to effect the transport of lipids through their circulation (for reviews see Ryan and Van der Horst, 2000; Van der Horst et al., 2001, Arrese et al., 2001; Van der Horst et al., 2002). This Lp, the protein component of which is homologous to that of LDL (Weers et al., 1993; Babin et al., 1999; Bogerd et al., 2000), was shown to extracellularly load specific lipids and to deliver them at target tissues. Thus, Lp was observed to take up lipids from the gut after dietary intake (Bauerfeind and Komnick, 1992), and was therefore presumed to be implicated in lipid supply to peripheral tissues, including lipid storage in the fat body. Remarkably, in insect species that engage in sustained flight activity, Lp is also involved and functions as a lipid shuttle, efficiently transporting lipids from the storage organ (fat body) to flight muscles (for recent review see Van der Horst et al., 2002). Even though Lp has the ability to deliver lipids extracellularly, without endocytosis and subsequent degradation of the lipoprotein, in *Locusta migratoria* a receptor homologous to LDLR was identified, that was expressed particularly in fat body and the brain (Dantuma et al. 1999). Experiments following transient expression of this insect receptor in a mammalian cell line revealed that it specifically mediates endocytosis of Lp, and was therefore denoted Lp receptor (LpR). A metabolic concept for the physiological function of this receptor, however, remained obscure.

To investigate the endocytic properties of this receptor in detail, we stably transfected endogenous LDLR-expressing Chinese hamster ovary (CHO) cells with an expression vector harboring wild-type (wt) *L. migratoria* LpR cDNA (Van Hoof et al., 2002). The studies described in **Chapter 2** evidence that LDL and Lp follow distinct intracellular routes after endocytosis mediated by LDLR and LpR, respectively (Table 1). Intracellular trafficking of fluorescently-labeled ligands in these cells was visualized with multicolor imaging and immunofluorescence. Shortly after receptor-mediated uptake, mammalian and insect lipoproteins colocalize in endocytic vesicles. In contrast to LDL, that is completely degraded in lysosomes after dissociating from its receptor (for reviews see Goldstein et al., 1985; Brown and Goldstein, 1986), both Lp and LpR evacuate from the endosomes, and converge in the endocytic recycling compartment (ERC; Maxfield and McGraw, 2004). Colocalization studies with transferrin (Tf) confirmed the identity of this organelle, via which internalized Tf recycles after receptor-mediated endocytosis by the Tf receptor (TfR; Yamashiro et al., 1984; Table 1). Similar to Tf, Lp is resecreted via the ERC, and exits the cell with a half-time of ~13 minutes. The ligand-recycling property of LpR *in vitro* is unique for

Table 1. Overview of recycling properties of wt receptors, mutants and hybrid receptors in different cell types.

Receptor	Cell type	Recycling of the receptor in the		Recycling of ligand
		absence of ligand	presence of ligand	
wt LDLR	CHO	yes	yes	no
	S2	not determined	not determined	no
$LDLR_{H562Y}$	CHO	yes	no	no
$LDLR_{H562N}$	CHO	yes	no	no
$LDLR_{1-292}LpR_{343-850}$	CHO	yes	no	no
LDLRΔEGF	CHO	no	no	no
wt LpR	CHO	yes	yes	yes
	S2	not determined	not determined	no
	fat body	not determined	not determined	probably
$LpR_{1-790}LDLR_{791-839}$	CHO	yes	yes	yes
$LpR_{1-342}LDLR_{293-839}$	CHO	no	no	no
wt TfR	CHO	yes	yes	yes
	S2	not determined	not determined	no
	Sf9	not determined	not determined	no

lipoprotein receptors. Thus far, all LDLR family members that have been shown to mediate endocytosis *in vitro* release bound ligand in sorting endosomes due to acidification of the vesicle lumen. The observation that LpR as well as Lp colocalize in the ERC strongly suggests that they form a stable receptor-ligand complex that remains intact despite the acidic environment. This implies that, in contrast to the LDLR-LDL complex, a decrease in pH does not induce ligand dissociation from LpR. If latter complex is stable at low pH, and LpR recycles normally, the ligand is carried along with the receptor to the cell surface.

Receptor-associated protein (RAP), which was shown to inhibit Lp endocytosis, is also transported to the ERC upon receptor-mediated endocytosis by LpR. In contrast to Lp, RAP was not completely resecreted. Approximately 50% of the total amount of internalized RAP remained visible in the cell for at least 1 h, which suggests that this fraction is not resecreted.

Irrespective of the endocytic properties of LpR *in vitro*, the occurrence of an endocytic receptor for Lp in the insect seems to conflict with the property of the ligand to selectively deliver lipids to target tissues in a non-endocytic manner. **Chapter 3** describes a putative role for LpR in *L. migratoria*. Western blot analysis revealed that LpR is temporally expressed in fat body tissue of young adults as well as larval locusts. Similar to mammalian cells (Van Hoof et al., 2002), fat body cells internalize fluorescently-labeled Lp and human RAP only when LpR is expressed. The receptor was shown to be down-regulated on the fourth day after an ecdysis. Although it cannot be excluded that fat body maintains the Lp-binding property at this stage, the tissue had lost its ability to internalize Lp. Subjecting adult locusts to starvation immediately after ecdysis resulted in prolonged LpR expression. In addition, starvation of adults after LpR had been down-regulated re-induced expression of the receptor. Because the fat body lipid depots are depleted after an energy-consuming process like ecdysis or starvation, the above studies suggest that endocytic uptake of Lp is only present when the fat body lipid depots are exhausted. The physiological role of endocytosis of Lp might thus be interpreted as providing an efficient mechanism to quickly replenish the fat body energy depots with lipids. In that case, it would be expected that expression of LpR might also

be induced after prolonged flight or oogenesis, as both processes require considerable amounts of lipids that are stored in the fat body. Alternatively, Lp endocytosis enables the fat body to obtain essential components that cannot be unloaded from Lp in the extracellular compartment, or cannot pass the plasma membrane by diffusion. Hydrocarbons and carotenoids are essential components for the properties and pigmentation of the insect cuticle, and are known to be transported in Lp through the insect body (for review see Soulages and Wells, 1994). Although part of these hydrocarbons may be produced in epidermal cells and oenocytes of the abdominal integument (Fan et al., 2003), starvation prevented hardening as well as pigmentation of the insect exoskeleton, which may be due to shortage of hydrocarbons and carotenoids derived from consumed plant material. Thus, the function of LpR as an endocytic receptor for Lp during the first few days after an ecdysis may relate to the uptake of essential components from the diet (e.g. hydrocarbons, carotenoids, phytosterols) that are less able to pass the cell membrane by diffusion.

LpR was shown to recycle bound ligand when expressed in a mammalian cell line (Table 1; Van Hoof et al., 2002). Whether LpR mediates recycling of Lp in fat body cells when endogenously expressed remained to be investigated. Little is known about receptor-mediated processes in insect cells. In **Chapter 4**, we initially determined the pathway of LDL and Tf in insect cell lines transfected with LDLR and TfR. After receptor-mediated endocytosis in mammalian cells, LDL is degraded whereas Tf is recycled (for review see Maxfield and McGraw, 2004). In contrast, both ligands have a similar fate upon internalization by *Drosophila* S2 cells transfected with LDLR and TfR (Table 1). In S2 transfectants that express LDLR and TfR simultaneously, LDL and Tf not only colocalize in endosomes immediately after endocytic uptake, but this situation is maintained also after a chase. This suggests that Tf remains in vesicles that contain LDL; the latter ligand is degraded in lysosomes after internalization by mammalian cells. Similar to the fate of Tf in S2 transfectants, Tf was retained in intracellular vesicles after endocytic uptake by *Spodoptera frugiperda* Sf9 transfectants expressing TfR (Table 1), suggesting that Tf is not recycled in these insect cells. To investigate whether the insect cell lines possess the ability to recycle Lp, S2 and Sf9 cells were transfected with LpR cDNA. Although Western blotting confirmed that LpR was expressed by both cell lines, only S2(LpR) transfectants appeared to take up fluorescently-labeled Lp. Whereas Lp is recycled after LpR-mediated endocytosis in mammalian cells, Lp was not resecreted from LpR-expressing S2 cells (Table 1), which implies that recycling is cell type specific. In contrast to the observations with insect cell lines, similar *in vitro* experiments with fat body tissue excised from locusts immediately after final ecdysis (Van Hoof et al., 2003) showed a significant decrease of Lp-containing vesicles that is indicative of recycling of Lp (Table 1). The remaining vesicles were, however, larger compared to those immediately after the incubation; the increase in size of which may be explained by fusion and maturation of endosomes into lysosomes. Thus, the process of Lp recycling in fat body cells appears to be less efficient than in mammalian cell lines.

The recycling of Lp in mammalian cells after LpR-mediated endocytosis is a remarkable phenomenon. Whereas mutations in the LDLR gene that impair ligand uncoupling result in degradation of the receptor-ligand complex (for reviews see Hobbs et al., 1990, 1992), wt LpR is not degraded in mammalian cells when in complex with ligand (Van Hoof et al., 2002). The LpR-mediated ligand recycling in mammalian cells could be explained by assuming that the luminal pH of mammalian endosomes after

entry of the receptor-ligand complex does not become as acidic as that of insect endosomes, and dissociation of the ligand from LpR does not occur. However, studies using TfR-transfected insect cell lines (Chapter 4) suggest that this is most likely not the case. The TfR-Tf complex is stable at very low pH (Dautry-Varsat et al., 1983), thus even if insect endosomes are more acidic than mammalian endosomes, Tf should recycle in complex with TfR. Because Tf was retained in insect cells and observed to colocalize with endocytosed LDL (Chapter 4), we conclude that the destination of a receptor-ligand complex is dependent on the type of cell that is used. Nonetheless, receptor type-specific domains also play a predominant role in determining the fate of the receptor either without, or in complex with ligand. Primary sequence homology analysis of multiple LDLR homologues conducted in **Chapter 5** reveals that putative motifs specific for lipoprotein receptor subfamilies are predominantly located in the intracellular domain. The intracellular C-terminus of *L. migratoria* LpR was replaced by that of human LDLR ($LpR_{1\text{-}790}LDLR_{791\text{-}839}$) to determine if the intracellular tail of LDLR induces degradation of a stable receptor-ligand complex. Similar to wt LpR (Van Hoof et al., 2002), the resulting hybrid receptor internalized and recycled Lp when expressed in CHO cells (Table 1). Apparently, substitution of the LpR intracellular tail by that of LDLR is not sufficient to elicit degradation of a ligand dissociation-deficient lipoprotein receptor. On the other hand, based on the homology analytical data, this property is not expected to reside in regions of the transmembrane and O-linked glycosylated domains of the receptor either. Therefore, a second hybrid receptor was generated, that was composed of the ligand binding domain of LpR and the region from epidermal growth factor (EGF) precursor homology domain to intracellular tail of LDLR ($LpR_{1\text{-}342}LDLR_{293\text{-}839}$). Fluorescence microscopy revealed that $LpR_{1\text{-}342}LDLR_{293\text{-}839}$ mediates endocytosis of Lp, which indicates that the function of the ligand binding domain is unaffected. However, in contrast to wt LpR and $LpR_{1\text{-}790}LDLR_{791\text{-}839}$, $LpR_{1\text{-}342}LDLR_{293\text{-}839}$ did not recycle (Table 1). Latter receptor has a fate that is similar to a ligand dissociation-deficient LDLR, the EGF domain of which was deleted (LDLRΔEGF; Davis et al., 1987; Table 1). These data imply that the EGF domain and intracellular tail of LDLR are involved in determining the destination of a receptor-ligand complex when ligand uncoupling is impaired.

The physiological role of the EGF domain of LDLR is to displace LDL from the ligand binding domain in endosomes (for reviews see Innerarity, 2002; Jeon and Blacklow, 2003). Familial hypercholesterolemia (FH) class 5 LDLR mutations are located in the LDLR EGF domain (e.g. H562Y), and are presumed to impair intracellular ligand dissociation (for reviews see Hobbs et al., 1990, 1992). Deletion of the EGF domain generates a receptor (LDLRΔEGF) that does not dissociate ligand [i.e. very-low-density lipoprotein (VLDL)] *in vitro* (Davis et al., 1987), and results in FH *in vivo* (Miyake et al., 1989). In **Chapter 6**, we studied the naturally occurring LDLR H562Y ($LDLR_{H562Y}$) FH mutation *in vitro*, and found that the mutant receptor recycles constitutively (Table 1). However, mimicking the physiological *in vivo* condition by extensive incubation of $LDLR_{H562Y}$-expressing cells with LDL resulted in an increase of receptor turnover. Even though LpR bears high sequence homology to LDLR, it recycles both without and in complex with bound ligand (Van Hoof et al., 2002). Tertiary structure analysis showed that His562 in LDLR corresponds to Asn643 in LpR. Substitution of His562 in LDLR by Asn does not sterically hindre surrounding residues in LDLR, and like His, Asn also contains an N atom in the delta position of the residue.

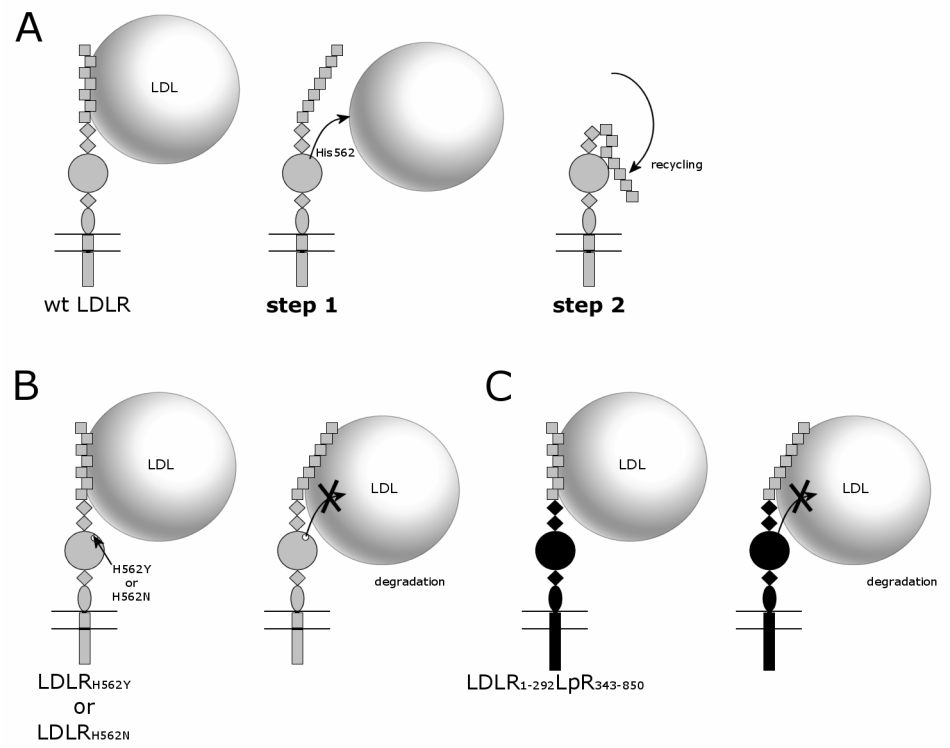

Figure 1. Schematic models of wild-type LDLR (A), LDLR$_{H562Y}$ or LDLR$_{H562N}$ (B), and LDLR$_{1-292}$LpR$_{343-850}$ (C), and the effect of acidification on the receptor-LDL complexes. LDL is dissociated from the ligand binding domain of LDLR by His562 at low pH (A, step 1). The ligand-free ligand binding domain folds onto the β-propeller of the EGF domain after which the receptor recycles to the cell surface (A, step 2). Substitution of the LDLR amino acid residue His562 by Tyr or Asn abolishes the ability of the mutated receptor to dissociate LDL at low pH; the receptor-LDL complex is not recycled, but degraded as a complex (B). Replacement of the LDLR region from EGF domain to intracellular tail by that of LpR has an effect similar to the H562Y and H562N mutations. LDLR$_{1-292}$LpR$_{343-850}$ is unable to uncouple LDL and is degraded as a receptor-LDL complex (C). LDLR domains are depicted in grey, and LpR domains in black. Squares, cysteine-rich repeats of the ligand binding domain; diamonds, EGF precursor-like repeats of the EGF domain; circle, β-propeller of the EGF domain; oval, O-linked glycosylation domain; short rectangle, transmembrane domain; long rectangle, intracellular tail.

The H562N mutation was expected to generate a receptor that is incapable of LDL dissociation, but able to recycle like the LpR-Lp complex. Yet, LDLR$_{H562N}$ did not recycle in complex with LDL. Instead, it was observed to behave like LDLR$_{H562Y}$ (Table 1). In addition to the two LDLR mutants, a hybrid receptor was constructed that harbors the ligand binding domain of LDLR, and the region from EGF domain to intracellular C-terminus of LpR. This LDLR$_{1-292}$LpR$_{343-850}$ mediates internalization of LDL, which indicates that the LDLR EGF domain is not essential for LDL binding. Similar to both LDLR mutants, LDLR$_{1-292}$LpR$_{343-850}$ recycles constitutively in the absence of ligand, and is degraded after LDL internalization (Table 1). Apparently, these mutations only affect ligand dissociation and not both ligand uncoupling and recycling of the receptors, like LDLRΔEGF. This suggests that, although it is

unanimously accepted that all classes of FH LDLR mutations lead to elevated plasma cholesterol levels *in vivo* (for reviews see Hobbs et al., 1990, 1992), class 5 mutations can be divided in two distinct subclasses: (1) mutations that impair ligand dissociation (e.g. $LDLR_{H562Y}$; Sun et al., 1994; J.C. Defesche, 2003, personal communication), and (2) those that prevent receptor recycling (e.g. LDLRΔEGF; Davis et al., 1987; Miyake et al., 1989).

The data presented in Chapter 6 strongly suggest that recycling of LDLR after ligand endocytosis is a process that occurs in discrete steps: (1) uncoupling of ligand from the ligand binding domain of LDLR, followed by (2) a conformational change of the LDLR ectodomain (Fig. 1A; Rudenko et al., 2002). His562 located in the β-propeller of the LDLR EGF domain is essential for ligand dissociation. Substitution of this amino acid residue by Tyr or Asn abolishes release of ligand from the ligand binding domain, resulting in degradation of the receptor-ligand complex (Fig. 1B). Tertiary structure analysis reveals that His562 of LDLR corresponds to Asn643 in the β-propeller of LpR. $LDLR_{1\text{-}292}LpR_{343\text{-}850}$ harbors the ligand binding domain of LDLR, and the EGF domain of LpR; it therefore also lacks this His residue. The hybrid receptor has a fate similar to $LDLR_{H562Y}$ and $LDLR_{H562N}$ (Fig. 1C), which is most likely due to their inability to release LDL in the endosome.

The three mutant receptors described above generally recycle in the absence of ligand. In contrast, LDLRΔEGF recycles neither constitutively (i.e. in the absence of ligand), nor in complex with ligand (i.e. VLDL). The receptor is unable to comply to the second condition required for receptor recycling, i.e. adopting a conformation in which the ligand binding domain is folded onto the β-propeller of the EGF domain (Fig. 2A). Similarly, the hybrid receptor $LpR_{1\text{-}342}LDLR_{293\text{-}839}$, composed of the ligand binding domain of LpR, and the region from EGF domain to intracellular tail of LDLR (Chapter 5), does not recycle in a ligand-free condition. The ligand binding domain of LpR is most likely not recognized by the EGF domain of LDLR; thus $LpR_{1\text{-}342}LDLR_{293\text{-}839}$ cannot change conformation, which is a prerequisite for recycling (Fig. 2B). Although all mutant receptors described above are most likely unable to uncouple ligand, $LDLR_{H562Y}$ and $LDLR_{H562N}$ (Fig. 2C), as well as $LDLR_{1\text{-}292}LpR_{343\text{-}850}$ (Fig. 2D) were observed to recycle normally in the absence of ligand. This suggests that these three mutant receptors can change their conformation in spite of the mutations. Moreover, the results suggest that in order to recycle, wild-type LpR also must adopt an arched conformation in the acidic endosome (Fig. 2E).

In contrast to the LDL-binding mutant receptors, wt LpR recycles in complex with either Lp or RAP when expressed in mammalian cells (Chapter 2). If contact between the ligand binding domain and the β-propeller of the EGF domain is required for recycling of the receptor, LpR-bound Lp does not hinder the conformational change. Possibly, Lp binds to cysteine-rich repeats that do not participate in stabilizing the interface between the ligand binding domain and the β-propeller of the folded receptor (Fig. 3A). RAP was observed to inhibit binding of Lp to LpR (Chapter 2), which suggests that both ligands bind to the same region of the receptor. In contrast to Lp, ~50% of the initial amount of RAP was recycled after LpR-mediated endocytosis. Quantitative studies using ^{125}I-labeled Lp and RAP suggest that the ligands bind to LpR in an approximately 1:4 molar ratio (Chapter 2). This indicates that 4 molecules of RAP bind to LpR, two of which are dissociated in the acidic milieu of the endosome lumen (Fig. 3B). In mammals, RAP assists in the folding of lipoprotein receptors, and binds

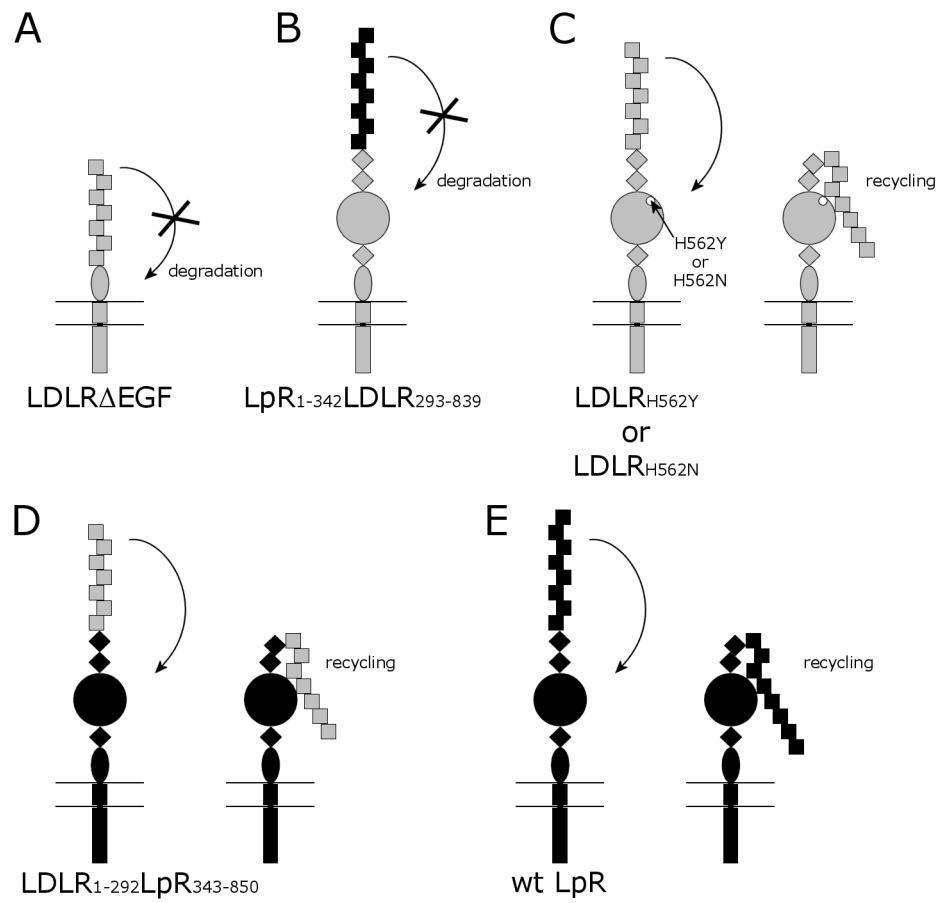

Figure 2. Schematic models of LDLRΔEGF (A), $LpR_{1-342}LDLR_{293-839}$ (B), $LDLR_{H562Y}$ or $LDLR_{H562N}$ (C), $LDLR_{1-292}LpR_{343-850}$ (D), and wild-type LpR (E), and the effect of acidification on the conformation of the receptors. LDLR?EGF cannot adopt an arched conformation due to the absence of the EGF domain and is degraded after internalization (A). The ligand binding domain of LpR fails to establish a solid connection with the EGF domain of LDLR. Similar to LDLRΔEGF, $LpR_{1-342}LDLR_{293-839}$ is not transferred to the ERC after endocytosis (B). Mutation of the LDLR amino acid residue His562 to Tyr or Asn does not affect the ability of the receptor to adopt a conformation in which the ligand binding domain is folded onto the β-propeller of the EGF domain; both mutant receptors recycle constitutively (C). In contrast to the reciprocal hybrid receptor, $LDLR_{1-292}LpR_{343-850}$ can form intramolecular bonds between the ligand binding domain of LDLR and the β-propeller of LpR, enabling the receptor to return to the cell surface after internalization (D). (E) LpR recycles after an acid-induced conformational change that is similar to that of LDLR. LDLR domains are depicted in grey, and LpR domains in black. Squares, cysteine-rich repeats of the ligand binding domain; diamonds, EGF precursor-like repeats of the EGF domain; circle, β-propeller of the EGF domain; oval, O-linked glycosylation domain; short rectangle, transmembrane domain; long rectangle, intracellular tail.

with high affinity to the ligand binding domain of many LDLR family members (Bu and Schwartz, 1998; Bu and Marzolo, 2000), including LpR (Van Hoof et al., 2002). Genomic sequence analysis reveals that RAP homologues are present in *D. melanogaster* and *A. gambiae* (Fig. 4), which suggests that RAP also functions as a folding chaperone for LpR in insects.

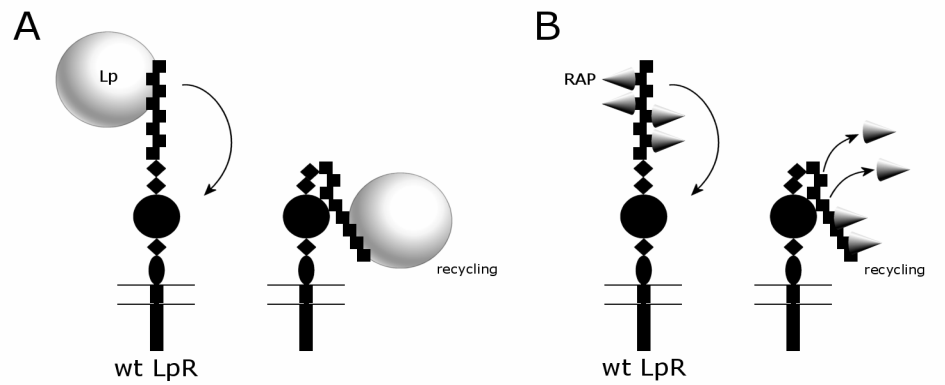

Figure 3. Schematic model of wild-type LpR, and the effect of acidification on the LpR-ligand complex. Lp remains attached to the ligand binding domain of LpR at low pH, and does not prevent the ligand binding domain to fold onto the β-propeller of the EGF domain; the LpR-Lp complex recycles to the cell surface (A). (B) LpR binds 4 RAP molecules that attach to different cysteine-rich repeats of the ligand binding domain. Two of these RAP molecules are dissociated upon acidificaton of the receptor milieu; the others remain attached to the ligand binding domain, and recycle in complex with LpR. LpR domains are depicted in black. Squares, cysteine-rich repeats of the ligand binding domain; diamonds, EGF precursor-like repeats of the EGF domain; circle, β-propeller of the EGF domain; oval, O-linked glycosylation domain; short rectangle, transmembrane domain; long rectangle, intracellular tail.

In *L. migratoria*, LpR mediates endocytosis of Lp in fat body cells (Chapter 3). Lipids derived from internalized Lp are presumably used to replenish the stores that become depleted after the energy-consuming process of ecdysis, or starvation. In other insects, LpR was found to be expressed in fat body, as well as oocytes (Cheon et al., 2001) and ovaries (Seo et al., 2003). Hemolymph proteins like Lp are internalized by oocytes and stored in large compartments that provide supplies for the developing insect embryo (Kulakosky and Telfer, 1990; Van Antwerpen et al., 1993). Although *L. migratoria* Lp was not taken up by Sf9 cells, which are derived from immature ovaries of *S. frugiperda* pupae (Vaughn et al., 1977), LpR-transfected S2 cells internalized Lp (Chapter 4). Similar to the observations in oocytes (Kulakosky and Telfer, 1990; Van Antwerpen et al., 1993), Lp was retained intracellularly in S2(LpR) transfectants. In contrast to the fate of Lp in insect cells, the ligand was recycled in CHO cells transfected with LpR (Van Hoof et al., 2002). Fat body tissue, expressing endogenous LpR, was shown to internalize Lp *in vitro*. Following a pulse of fluorescently-labeled Lp, extensive incubation in growth medium without Lp resulted in a significant decrease in the number of fluorescently-labeled Lp-containing vesicles (Chapter 4). This suggests that the ligand is resecreted, albeit not as efficiently as from CHO(LpR) cells. Apparently, the fate of a ligand is not solely determined by the receptor to which it is attached, but also depends on the type of cell expressing the receptor. Expression of other lipoprotein receptor hybrids and mutants in various cell types will extend the knowledge of receptor properties *in vitro* as well as *in vivo*. Novel insights into LDLR functioning may eventually lead to the development of specialized treatments for the different classes of FH LDLR mutations.

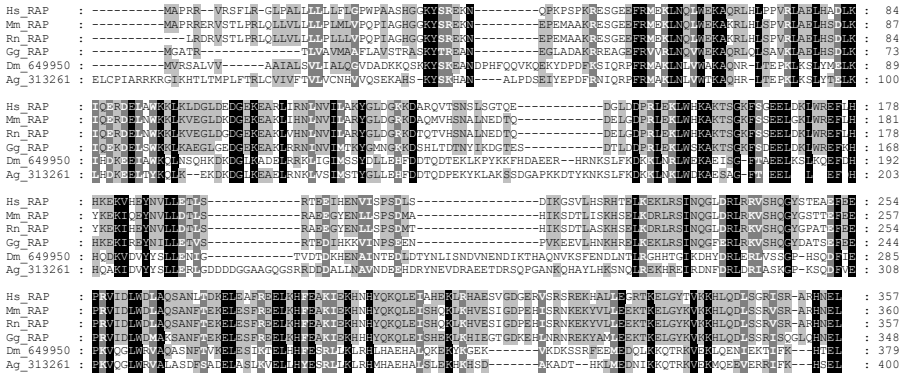

Figure 4. Manually modified Clustal-W amino acid multiple sequence alignment of RAP homologues. Amino acid sequences of the species used were obtained with NCBI Entrez Protein search engine (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Protein). Alignment of the sequences was created with the on-line CMBI Clustal-W server (http://www.cmbi.kun.nl/bioinf/tools/clustalw.shtml) using BLOSUM 30 (gap open penalty 10.00, and gap extension penalty 0.05). Hs_RAP, *Homo sapiens* RAP (accession number NP_002328); Mm_RAP, *Mus musculus* RAP (accession number NP_038615); Rn_RAP, *Rattus norvegicus* RAP (accession number Q99068); Gg_RAP, *Gallus gallus* RAP (accession number CAA05085); Dm_649950, *Drosophila melanogaster* annotated RAP (putative; accession number NP_649950); Ag_313261, *Anopheles gambiae* annotated RAP (putative; accession number XP_313261). Black boxes with white characters show residues that are identical in all species; dark grey boxes with white characters show residues that are similar in all species; light grey boxes with black characters show residues that are similar in at least four of the six species.

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SAMENVATTING IN HET NEDERLANDS

Lipiden (vetten) vormen een belangrijke bron van energie voor tal van lichamelijke activiteiten. De lipiden die zijn opgeslagen in vetweefsel in het lichaam worden verbrand in de spieren tijdens langdurige inspanning. Via de lipiden uit het voedsel (maar ook door omzetting van opgenomen koolhydraten in vetten) kunnen de lipidreserves in het vetweefsel weer worden aangevuld. De lipiden uit het voedsel worden in het bloed van de darm naar het vetweefsel getransporteerd. Omdat lipiden slecht oplosbaar zijn in waterige vloeistoffen (zoals bloed) maakt het lichaam gebruik van speciale eiwitten om de lipiden te verschepen. Deze eiwit-lipidcomplexen (lipoproteïnen), bestaande uit één of meerdere eiwitten (apolipoproteïnen) en vele typen lipiden, komen in zoogdieren (zoals de mens) in veel verschillende vormen voor. De diverse soorten lipoproteïnen hebben specifieke samenstellingen, functies en mechanismen waarmee de lipidvracht wordt overgedragen aan de weefsels.

In sommige gevallen wordt slechts een deel van de lipidvracht buiten de cel van het lipoproteïne afgeladen (Hoofdstuk 1, Fig. 1). De door darmcellen geproduceerde lipoproteïnen (chylomicronen) kunnen tijdelijk aan vetcellen binden die er lipiden aan onttrekken welke vervolgens worden opgeslagen in compartimenten in de vetcellen. VLDL is een ander lipoproteïne en wordt geproduceerd door de lever. Net als chylomicronen kunnen VLDL partikels een deel van hun lipiden afgeven aan vetweefsel. Tijdens de hierboven beschreven processen fungeert het lipoproteïne HDL als donor en acceptor voor sommige eiwitcomponenten en lipiden van chylomicronen en VLDL. Door de eiwit- en lipiduitwisseling worden de chylomicronen en VLDL partikels omgezet in respectievelijk chylomicron-overblijfselen en LDL partikels. Deze laatstgenoemde deeltjes hebben in vergelijking met andere lipoproteïnen een relatief hoge concentratie aan cholesterol. Een te hoge concentratie cholesterol in het bloed (hypercholesterolemie) vergroot de kans op hart- en vaatziekten. De hoeveelheid LDL in het bloed moet dan ook binnen bepaalde (vrij lage) waarden worden gehouden (plasma cholesterol homeostase).

In contrast met de hierboven beschreven processen waarbij lipiden buiten de vetcel van de lipoproteïnen worden afgeladen, worden de chylomicron-overblijfselen en LDL partikels door de levercellen opgenomen (Hoofdstuk 1, Fig. 1). De opname (endocytose) wordt gemedieerd door receptoren. Het proces van LDL opname is uitgebreid onderzocht en goed gekarakteriseerd. De LDL receptor (LDLR) is een eiwit dat door de membraan (buitenwand) van de cel heen steekt en met het extracellulaire (buiten de cel gelegen) deel een in het bloed circulerend LDL partikel kan binden (Hoofdstuk 1, Fig. 8). Het LDLR-LDL complex wordt vervolgens in zijn geheel door de cel naar binnen gehaald door instulping en afsnoering van de membraan (receptor-gemedieerde endocytose; Hoofdstuk 1, Fig. 6). Het milieu in het ontstane intracellulaire (in de cel gelegen) blaasje (endosoom) wordt nu zuurder; de verzuring in het endosoom veroorzaakt de ontkoppeling van het LDLR-LDL complex. Delen van de endosoommembraan met daarin LDLR stulpen uit en worden afgesnoerd van het endosoom. Het endosoom met daarin LDL verandert in een lysosoom, waarin de resterende deeltjes volledig worden afgebroken, terwijl de afgesnoerde blaasjes met

LDLR naar een ander celcompartiment (organel) worden getransporteerd. De blaasjes fuseren met dit speciale organel, het endocytisch recycling compartiment (ERC), waarvandaan allerlei geïnternaliseerde (membraan)eiwitten worden teruggebracht naar de celmembraan. Kort samengevat bindt LDL aan LDLR waarna het complex de cel in wordt gebracht. In de cel wordt het LDL losgekoppeld van de receptor; het LDL wordt afgebroken en LDLR wordt teruggetransporteerd naar de celmembraan, en kan een nieuw LDL deeltje binden en internaliseren. Iedere LDLR ondergaat deze cyclus (Hoofdstuk 1, Fig. 6) gemiddeld 150 keer alvorens te worden afgebroken. Ook in afwezigheid van LDL wordt LDLR continu geïnternaliseerd en gerecycled.

In tegenstelling tot zoogdieren maken insecten gebruik van slechts één soort lipoproteïne dat lipoforine (Lp) wordt genoemd. Alle tot nu toe beschreven processen waarbij Lp fungeert als lipidtransporteur vinden plaats buiten de cel (Hoofdstuk 1, Fig. 2). Het lipidopslagorgaan in insecten (het vetlichaam) produceert grote hoeveelheden Lp. De in het insectenbloed (hemolymfe) circulerende Lp partikels geven een deel van hun lipidvracht af aan organen die daar behoefte aan hebben, waarna de lipidarme Lp deeltjes hun voorraad aanvullen met lipiden afkomstig uit het vetlichaam en andere organen. Een goed model voor deze processen is de Afrikaanse treksprinkhaan, *Locusta migratoria* (Hoofdstuk 1, Fig. 3), een beruchte veelvraat die in grote zwermen voor kan komen. Het insect kan wel 10 uur ononderbroken vliegen en is daarmee in staat om zeer lange afstanden af te leggen. Vliegen is het meest energievergende proces dat bekend is in de natuur. Om dat langdurig te kunnen volhouden moet het insect erg zuinig en efficiënt omgaan met de Lp partikels die het transport van lipiden van het vetlichaam naar de vliegspieren mogelijk maken. Ook bij dit proces vindt er geen opname en afbraak van het volledige lipoproteïne-deeltje plaats, maar slechts de extracellulaire gedeeltelijke ontlading en overdracht van de lipidvracht van Lp naar de vliegspieren (Hoofdstuk 1, Fig. 4).

Afgezien van de hierboven beschreven processen is Lp ook betrokken bij lipid transport van de darm naar het vetlichaam na de opname van voedsel. De belading van circulerend Lp met lipiden afkomstig uit de darm is een extracellulair proces. Echter, over het opnamemechanisme van Lp-afkomstige lipiden door het vetlichaam was nog nauwelijks iets bekend. De verwachting was dat dit proces ook buiten de vetlichaamcellen zou plaatsvinden, maar de ontdekking van een LDLR-achtige insectenreceptor die wordt geproduceerd door vetlichaamcellen suggereerde dat Lp ook kan worden geëndocyteerd. Het onderzoek gepresenteerd in dit proefschrift beschrijft de manier waarop deze insecten Lp receptor (LpR) functioneert. De hieruit voortkomende resultaten geven tevens inzicht in het functioneren van LDLR en bieden de mogelijkheid om een specifieke vorm van familiaire hypercholesterolemie (FH), een erfelijke aandoening waarbij LDLR niet naar behoren functioneert, beter te begrijpen.

In **Hoofdstuk 2** hebben we gebruik gemaakt van in kweekflessen groeiende zoogdiercellen [Chinese hamster ovarium (CHO) cellen] die van nature LDLR produceren. Met behulp van DNA cloneringstechnieken hebben we deze CHO cellen, naast LDLR, ook LpR laten produceren. Tevens werden menselijk LDL en *L. migratoria* Lp met respectievelijk een rood en een groen fluorescent label gekleurd. Door deze fluorescent-gelabelde zoogdier- en insecten lipoproteïnen gelijktijdig aan de CHO cellen aan te bieden konden met behulp van een fluorescentiemicroscoop de opname en intracellulaire distributie van beide deeltjes gelijktijdig worden bekeken en vergeleken. Uit dit onderzoek blijkt dat LDL en Lp specifiek door respectievelijk LDLR en LpR worden geïnternaliseerd in de cellen. Zoals verwacht bleef LDL na opname in

de cellen achter. Echter, Lp verdween geleidelijk uit de cel; dit lipoproteïne werd na opname weer uitgescheiden. Dus zoogdier- en insecten lipoproteïnen hebben een verschillend lot na receptor-gemedieerde opname door zoogdiercellen.

Tijdens de ontwikkeling van een jong tot volwassen insect ondergaat *L. migratoria* vijf verschillende vervellingen. Na de vijfde vervelling is het insect volwassen en verandert niet meer van uiterlijk. De aanwezigheid van LpR in het insect was tot dan toe slechts indirect aangetoond in volwassen *L. migratoria* door het zichtbaar maken van de erfelijke informatie voor dit eiwit. Om te bepalen of het receptor-eiwit ook daadwerkelijk wordt geproduceerd door het vetlichaam van *L. migratoria* hebben we in **Hoofdstuk 3** antistoffen gebruikt om LpR aan te kleuren in extracten van vetlichaamcellen. Hieruit bleek dat LpR alleen aanwezig is gedurende de eerste vier dagen na een vervelling, een energievergend proces waarbij veel lipiden uit het vetlichaam worden verbruikt. De internaliserende capaciteit van LpR in vetlichaamcellen werd onderzocht op een zelfde manier als beschreven in Hoofdstuk 2: vetlichaam werd geïsoleerd uit *L. migratoria* van verschillende leeftijden, waarna de weefsels werden gedrenkt in een oplossing met fluorescent-gelabeld Lp. Fluorescentiemicroscopische analyse toonde aan dat Lp alleen wordt geïnternaliseerd door weefsel dat LpR produceert (dus vetlichaam dat is geïsoleerd uit sprinkhanen binnen 5 dagen na een vervelling). De afname van LpR productie kon worden uitgesteld door de insecten meteen na een vervelling te hongeren, en kon worden geïnduceerd door de insecten vanaf de vijfde dag na een vervelling te hongeren. Dit wijst erop dat LpR betrokken is bij de opname en opslag van lipiden afkomstig van Lp.

Uit Hoofdstuk 2 blijkt dat LpR in zoogdiercellen de recycling van Lp medieert. Om te bepalen of het lot van Lp in insectencellen gelijk is aan dat in zoogdiercellen hebben we in **Hoofdstuk 4** gebruik gemaakt van in kweekflessen opgegroeide insectencellen. Omdat de intracellulaire routes van opgenomen eiwitten in deze cellen nog niet gekarakteriseerd waren hebben we eerst geprobeerd deze wegen te definiëren met behulp van menselijk LDL en menselijk transferrine (Tf). Tf is een eiwit dat in zoogdiercellen wordt opgenomen door de Tf receptor (TfR). In tegenstelling tot menselijk LDL wordt het TfR-Tf complex, net zoals het insecten LpR-Lp complex in CHO cellen, als geheel gerecycled via het ERC. In zoogdiercellen worden menselijk LDL en menselijk Tf dan ook veelvuldig gebruikt als markers om de beide routes (de afbraak- en recyclingroute) aan te kleuren. In Hoofdstuk 4 beschrijven we het gebruik van insectencellen die zodanig genetisch gemodificeerd zijn dat ze menselijk LDLR en TfR produceren. Fluorescent-gelabeld LDL en Tf werden tegelijkertijd aan deze cellen aangeboden welke daarna werden geanalyseerd met een fluorescentiemicroscoop. In tegenstelling tot de divergente routes die LDL en Tf in zoogdiercellen volgen bleken beide eiwitten in insectencellen te worden opgeslagen in intracellulaire blaasjes. Lp geïnternaliseerd door LpR producerende insectencellen werd ook niet uitgescheiden. Echter, vergelijkbare experimenten met andere insectencellen (vetlichaamcellen) lieten zien dat uit deze cellen wèl een grote hoeveelheid van het opgenomen Lp verdwijnt na LpR-gemedieerde opname. Uit deze gegevens kan worden geconcludeerd dat het lot van een lipoproteïne (of ander eiwit, zoals Tf), niet alleen wordt bepaald door de receptor, maar ook afhankelijk is van het celtype dat de receptor produceert. Dit suggereert dat de cel een (deel van de) receptor moet herkennen om te weten wat er met het daaraan gekoppelde eiwit moet gebeuren. Het meest voor de hand liggende receptor fragment dat voor deze herkenning kan zorgen is het intracellulaire deel omdat dit direct in contact staat met de rest van de cel.

In **Hoofdstuk 5** proberen we te achterhalen of het intracellulaire deel van LDLR bepaalt waar het aan de receptor gebonden eiwit (in dit geval LDL) uiteindelijk terechtkomt. Hiervoor hebben we een hybride receptor gemaakt waarvan het extracellulaire deel afkomstig is van LpR en het intracellulaire deel van LDLR. In CHO cellen was de resulterende receptor in staat om Lp te endocyteren en bleek het eiwit te recyclen zoals de normale LpR dat ook doet. Dit suggereert dat de introductie van het intracellulaire deel van LDLR in LpR niet voldoende is om het lot van Lp te veranderen na opname door de receptor. Daarom hebben we de hybriden-aanpak uitgebreid met de constructie van een tweede hybride. Deze receptor bevatte alleen nog het gedeelte van LpR waarmee het Lp kan binden; de rest was afkomstig van LDLR. Hoewel deze hybride receptor wel zorgde voor Lp-opname was de receptor niet in staat om, net zoals de normale LDLR, te recyclen. Dit suggereert dat deze tweede hybride receptor vlak na internalisatie wordt afgebroken in lysosomen. Sommige van de vele genetische afwijkingen die het normale functioneren van LDLR verstoren hebben tot gevolg dat de receptor niet meer in staat is om normaal te recyclen, en leiden tot de bovenbeschreven aandoening FH. Recycling-gestoorde receptoren behoren tot FH klasse 5 mutanten; het onvermogen van deze mutante receptoren om te recyclen is meestal het gevolg van het niet kunnen ontkoppelen van gebonden LDL in de endosomen. Hoewel de normale LpR niet wordt afgebroken ondanks dat Lp in endosomen aan de receptor gebonden blijft, lijkt het onvermogen van LpR om Lp te ontkoppelen ook enigszins op de FH klasse 5 mutatie. Drie-dimensionale structuur analyse van LDLR en LpR wijst uit dat LpR op heel specifieke plaatsen verschilt van de normale LDLR, en dat één van deze plaatselijke afwijkingen in LpR overeenkomt met een afwijking in een FH klasse 5 mutante LDLR.

In **Hoofdstuk 6** hebben we de hierboven beschreven mutante LDLR zoals die voorkomt in de patiënt nagemaakt in het laboratorium en laten deze door CHO cellen produceren om het afwijkende gedrag op een cellulair niveau met fluorescentiemicroscopie te kunnen bestuderen. Tevens construeren we een tweede mutante receptor waarbij hetzelfde specifieke LDLR stukje is vervangen door dat van LpR, en maken we een derde hybride receptor. Deze laatste hybride receptor heeft het gedeelte waarmee LDL wordt gebonden van LDLR en de rest is afkomstig van LpR (en is daarmee de reciproke hybride van de tweede hybride receptor beschreven in Hoofdstuk 5). Alledrie de veranderde receptoren bleken LDL normaal te kunnen internaliseren wanneer ze door CHO cellen werden geproduceerd. Ook bleken ze alledrie in staat om te recyclen in afwezigheid van LDL. Echter, na binding van LDL kwamen geen van de drie receptoren meer terecht in het ERC, wat er op duidt dat deze LDL-bindende receptoren niet kunnen recyclen in aanwezigheid van LDL. Het verschil met de in Hoofdstuk 5 beschreven recycling-gestoorde receptor (de tweede hybride receptor) is dat deze in zowel vrije, als in Lp-gebonden toestand niet kon recyclen. De in Hoofdstuk 6 beschreven mutante en hybride receptoren konden wel in vrije vorm recyclen, maar hebben na binding van LDL waarschijnlijk hetzelfde lot als het gebonden lipoproteïne (LDL), dat na opname in lysosomen wordt afgebroken. Hieruit kan worden geconcludeerd dat FH klasse 5 mutaties niet allemaal leiden tot dezelfde afwijking in het lot van de receptor. Het verschil is klaarblijkelijk subtieler: sommige FH klasse 5 mutaties zorgen ervoor dat de receptor kan recyclen in afwezigheid van LDL, maar wordt afgebroken in aanwezigheid van LDL, terwijl andere mutaties leiden tot afbraak van de mutante LDLR ongeacht de aan- of afwezigheid van LDL.

De analyse en karakterisatie van LpR hebben bijgedragen aan de uitbreiding van het inzicht in het functioneren van de menselijke LDLR. Tevens hebben de gegevens over de insecten receptor en verschillende LDLR mutanten sterke aanwijzingen geleverd voor een onderverdeling van FH klasse 5 mutaties. Het gebruik van relatief eenvoudige modelorganismen zoals een insect, waarin processen als lipid transport op een wat andere manier plaatsvinden, biedt dan ook uitstekende mogelijkheden om de ingewikkelde processen in zoogdieren zoals de mens te bestuderen, en kunnen leiden tot nieuwe inzichten en ideeën over ziekten en afwijkingen in de mens.

Promoveren is als één grote bungee jump. Vantevoren weet je eigenlijk niet zo goed waar je aan begint. Iedereen zegt dat je het absoluut moet doen, dus vooruit dan maar. Als je dan eenmaal de "sprong in het diepe" waagt is er geen weg terug. Het eindigt veel sneller dan je had verwacht. Met het zweet op je voorhoofd vraag je je af of je zo blij bent omdat je er ondertussen van hebt kunnen genieten...
Of omdat het allemaal achter de rug is.

DANKWOORD

Zo... Het schrijven van dit boekje en al het laboratoriumwerk dat daar voor nodig was viel niet mee. Maar het is me ook zeker niet tegengevallen! Hoewel het Kruytgebouw niet het mooiste gebouw van de Uithof is (en dat is een understatement) is het min of meer een tweede thuis voor me geworden. Wat heb ik daar de afgelopen 4½ jaar nou eigenlijk allemaal zitten doen?

Iedere dag vroeg opstaan, want er ligt altijd meer werk te wachten dan je in een normale werkdag gedaan krijgt. Om 8:03 uur de deur van de schrijfkamer van het slot en jas aan de kapstok. Even kijken wat er ook al weer op de planning stond. Oh ja, eerst die labelingsreactie inzetten. Dan heb ik daarna 45 minuten de tijd om vast een deel van het celkweekwerk te doen. Hmm, die 06-lijn wil nog niet goed groeien; straks even overleggen met Kees. De immunoblot dus maar uitstellen tot volgende week. Kunnen de sprinkhanen die ik apart had gezet ook weer terug naar hun vriendjes in de klimaatkamer. Tijd om de gelabelde eiwitten te zuiveren en de concentratie te bepalen.

Jan: "Môge, Dennis."

Dennis: "Môge, Jan."

Jan: "Kom je weer zo'n gigantische hoeveelheid eiwitbepalingen doen?" (Jan doet er meestal meer dan 50)

Dennis: "Yep! Deze keer drie in plaats van twee, want ik neem humaan RAP ook mee."

Jan: "Tjongejonge... Kijk je wel uit dat je er niet teveel doet?"

Dennis: "Daar heb ik jou toch voor." *grijns*

Tien minuutjes incuberen; kan ik mooi even e-mail checken. Ah, da's mooi, de aangevraagde artikelen zijn af te halen bij de FSB bibliotheek. En die post-doc in de USA wil wel enkele tientallen microliters van dat antilichaam opsturen. Nu nog een mailtje naar die groep in Zuid Afrika sturen om te vragen of ze ook receptor-bindingstudies hebben gedaan met hun FH mutanten.

Jana: "Dobre rano, Dennis."

Dennis: "Dobre rano, Jana. Hoe staat het met die hybriden?"

Jana: "Die heb ik vanmiddag klaar. In welke concentratie wil je het DNA hebben?"

Dennis: "Rond de 1½ microgram per microliter zou mooi zijn. Leg het epje maar op mijn bureau, dan kan ik vanmiddag meteen de transfecties inzetten."

Verder met de eiwitbepaling. Daarna de vijf 12-wells platen met cellijnen uit de stoof en de geplande endocytose assay uitvoeren.

Dennis: "Môge, Kees."

Kees: "Hoi."

Dennis: "Zeg, Kees, ik zat te denken dat als ik die i/L-hybride nou eerst 5 minuten incubeer met OG-RAP en een overmaat aan HDLp in aanwezigheid van monensine bij 37°C, het pulse medium vervang door kweekmedium met daarin een twee keer zo hoge concentratie nocodazole als de vorige keer en dan eerst humaan LDL toevoeg zonder HEPES 50 waarna ik de temperatuur verlaag tot 4°C, de cellen was met PBS en dan weer overzet in medium met 2189/90 antilichaam, maar nu zonder chloroquine, dan kan ik met C7 of 121 kijken of de receptor na 20 minuten colocaliseert met Tf in het ERC!"

Kees: "..."

Dennis: "Maar ik moet weer verder, want mijn cellen schreeuwen om aandacht."

Tijd om de cellen te fixeren en een immunofluorescentie assay er achteraan om de receptoren zichtbaar te maken.

Karine: "Bonjour Dennies."

Dennis: "Bonjour Karien. Weet je al wat die apoLp-II dubbelband is die je in de eiwitgel ziet?"

Karine: "Nee, maar we denken aan glycosylering."

Dennis: "Het heeft vast iets te maken met de herkenning van het ligand door de receptor."

Karine: "Yeah, sure!" *grijns*

Incubatie met het primaire antilichaam ingezet om 12:08; mooie tijd voor koffiepauze. Ondertussen even een mailtje naar Marcel sturen over die Nature publicatie.

Dick: "Hoi Dennis, denk je nog aan het stukje dat je zou schrijven?"

Dennis: "..."

Dick: "Voor het IB...?"

Dennis: "Oh, ja! Ik leg het vóór vijf uur op je bureau."

Dick: "Heb je ondertussen even tijd om me te laten zien hoe je ook al weer de achtergrond van een figuur transparant kon maken in PowerPoint?"

Dennis: "Tuurlijk." *klick ... klick klick klick ... klick klick ... klick klick klick*

Dick: "Ach, wat ben je toch handig met die computers."

Dennis: "Och... Hé, ik moet weer verder met mijn experimentjes!"

Secundaire antilichaam toevoegen en precies 20 minuten om te lunchen. Na de lunch zie ik het epje van Jana op mijn bureau. Eerst nog even e-mail lezen voordat ik de transfecties inzet. Mailtje van Marcel met de tekst "check!" als reactie op mijn mail over de Nature publicatie. Na de transfectie de cellen van het endocytose experiment inbedden in mowiol en met Kees overleggen wat we moeten doen met die 06-lijn die maar niet wil groeien.

Kees: "Gewoon een nieuwe stock uit de -80°C in kweek zetten. En wat betreft dat experiment waar je het vanochtend over had: misschien moet je ook maar de monoclonale lijn meenemen als controle."

Goed idee. Nog een uur om dat stukje voor het IB bij Dick neer te leggen en dan de cellen analyseren met de confocale laser microscoop.

Dennis: "Hier is het stuk voor het IB, Dick."

Dick: "Ach, maar je bent goed!"

Snel met mijn preparaten naar de 3^{e} verdieping waar de microscoop staat. Helaas, de microscoop is bezet tot 19:00 uur. Dat betekent dat ik vanavond terug moet komen; de analyse van alle preparaten kost me wel een paar uur. Dan maar die artikeltjes ophalen bij de bibliotheek. Na het lezen ervan blijkt het alweer half zeven te zijn geweest. De tijd vliegt (als je plezier hebt)!

Misschien blijkt het niet uit de hierboven beschreven (normale) werkdag, maar ik heb ontzettend veel te danken gehad aan iedereen om me heen. Zonder de vele collega's op de werkvloer had ik dit boekje nooit kunnen schrijven. **Dick van der Horst**, ik wil jou in het bijzonder bedanken voor de mogelijkheid om in jouw groep het onderzoek te kunnen doen dat in dit proefschrift beschreven staat. Je hebt me alle vrijheid gegeven die ik gedurende het onderzoek nodig dacht te hebben en mijn Engels is er ontzettend op vooruit gegaan. Niet alleen jou, maar ook **Kees Rodenburg** wil ik hartelijk

bedanken voor de vele inspirerende gesprekken die niet alleen maar van wetenschappelijke aard zijn geweest. Kees, een betere kamergenoot had ik niet kunnen hebben.

Verder wil ik alle anderen van de Leerstoelgroep Stofwisselingsfysiologie bedanken voor de hulp en natuurlijk ook de gezelligheid gedurende de afgelopen jaren: **Jan van Doorn**, **Wil van Marrewijk**, **Marcelle Kasperaitis**, **Marcel Smolenaars**, **Karine Valentijn**, **Jacques Diederen**, **Rob Oudejans**, **Masja van Oort** en **Yvonne Derks**. **Jana Kerver**, in het bijzonder wil ik jou bedanken voor de gedrevenheid waarmee je altijd in no-time de constructen voor me klaar had. En **Sigrid Roosendaal**, jou wil ik niet alleen bedanken maar ook veel succes wensen met de voortzetting van het onderzoek.

Ook de studenten die ik onder mijn hoede heb gehad hebben een leerzame bijdrage geleverd. **Salim Volger**, **Jens Dirkse**, **André Dales** en **Jurjen van Bolhuis**, ik hoop dat jullie veel hebben geleerd en een idee hebben van hoe het is om met twee voeten in de onderzoekswereld te staan. Jullie stages waren voor mij in ieder geval een leerzaam proces!

Milena Stosic, I thank you for your hard work in our lab. The acknowledgement in the Journal of Lipid Research article is proof of your fruitful efforts. Keep up the good work and I am sure you will become an excellent scientist!

Dan zijn er natuurlijk onze verdieping-genoten van wie ik er drie (of zijn het er vier?) in het bijzonder wil noemen: **Jan Bogerd**, mijn mentor, en **Joke Granneman** die mij zelfs "onderdak" heeft geboden toen de Westvleugel werd afgesloten wegens de beruchte brand! En **Hans van Aken**, bedankt voor het sequencen van alle constructen. **Thomas Schulz**, je was dan wel geen verdieping-genoot, maar je deed wel heel erg je best door veel in onze gang op en neer te lopen. En dat gaf mij iederekeer weer de gelegenheid om even wat technische gegevens en praktische details aan je te vragen. Ook bedankt voor het beschikbaar stellen van het celkweek lab op jullie afdeling na de brand.

Ook heb ik veel te danken aan alle leden van het Instituut voor Biomembranen van wie ik veel heb geleerd en materialen heb ontvangen. **Tobias Dansen**, **Arjan de Brouwer**, **Dennis van der Vlies** en **Mika Ruonala** voor alle microscopische technieken die jullie me hebben bijgebracht. **Ineke Braakman**, **Annemieke Jansens** en **Jürgen Gent**, omdat julllie ook aan de LDL receptor werk(t)en heb ik veel primaire antilichamen van jullie kunnen krijgen die me de mogelijkheid gaven om een paar prachtige publicaties te schrijven. **Peter van der Sluijs**, bedankt voor de TfR constructen en de kritische blik die je regelmatig op mijn werk wierp. **Irina Sorokina**, jouw wil ik niet alleen bedanken voor de secundaire (fluorescente) antilichamen, maar natuurlijk ook voor de leuke gesprekken buiten het werk. Eigenlijk zou ik iedereen die ik binnen het Instituut voor Biomembranen heb leren kennen moeten bedanken. Toch zijn er een paar die ik extra wil bedanken. **Jean-Luc Murk**, uit de wederzijdse interesse in het werk dat we deden zijn veel stimulerende gesprekken ontstaan. Ik heb erg veel aan je kennis en inzichten gehad. **Martin Sachse**, ik bedank je in het Nederlands; mijn Duits is nog steeds niets vergeleken met jouw Nederlands. Het Engels gebruikten we voornamelijk om over elkaars onderzoeksonderwerpen te kunnen praten en dat is in dit geval niet van toepassing. Ik dank alle AiO's van het instituut voor Biomembranen voor de gezelligheid en leuke AiO-dagen, AiO-avonden en AiO-retraites die ik heb mogen meemaken. Even een aantal inside-jokes: **Hester van Heusden**, bezoek zoveel mogelijk saaie lezingen want die bezorgen je een bron van artistieke creativiteit; **Claudia van**

Tiel, getuige van de verleidelijke heupbewegingen van buikdanseressen in Istanbul; **Rinse Klooster**, een op hol geslagen paard, een automatische hectometerpaaltjes-schoonmaakmachine, wat wordt het volgend jaar?; **Claudia Fila**, you gave a nice "twist" to the AiO-retreat of 2003, next time I'll try to be more "flexible."

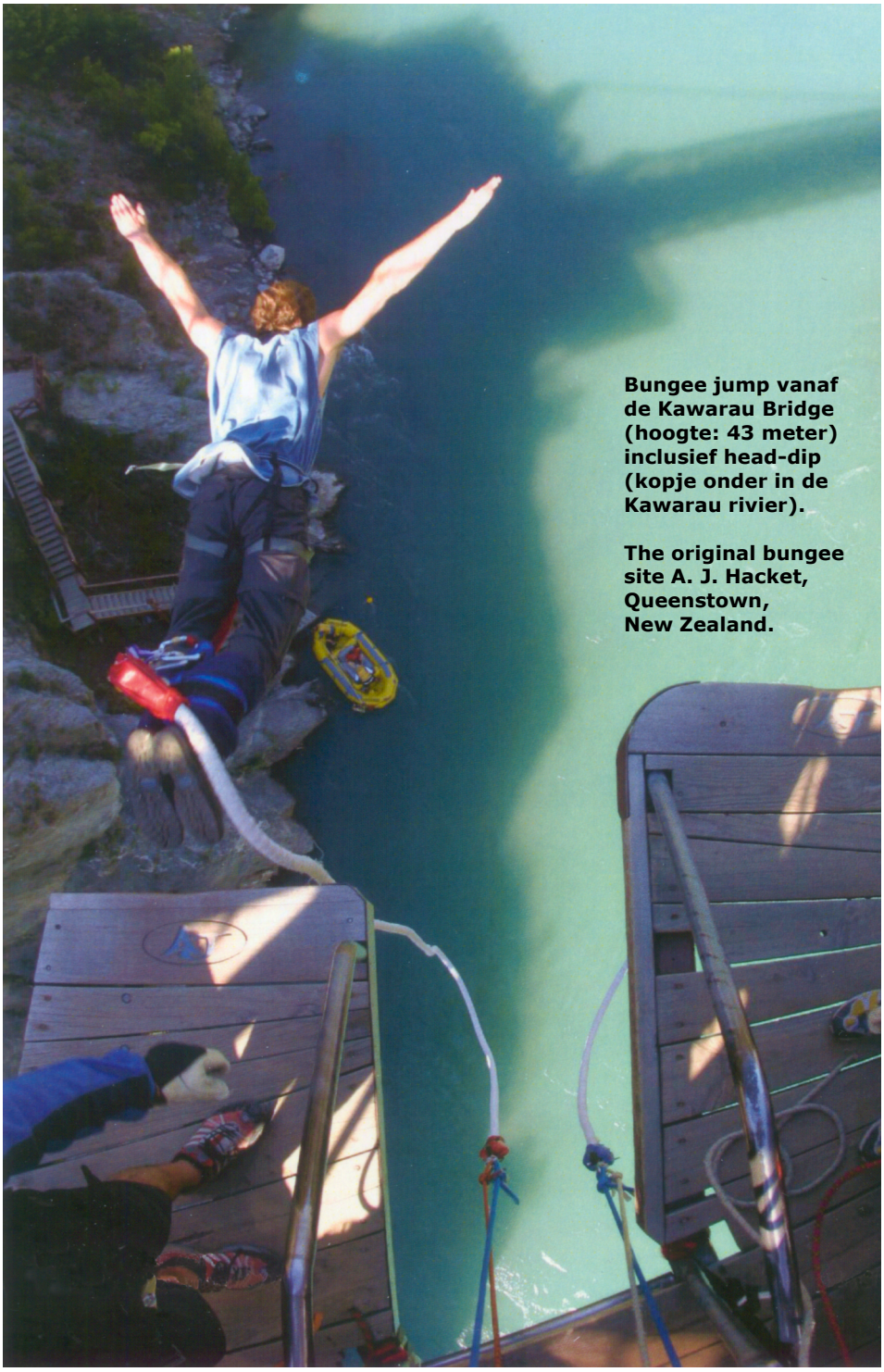
Ook wil ik het niet-wetenschappelijke personeel van de Universiteit Utrecht bedanken. **Cor Nijhoff** en **Ton van Domselaar**, het is dan eindelijk zover: Meer kennis met Dennis! **Frits Kindt**, **Ronald Leitho**, **Piet Brouwer** en **Wil van Veenendaal**, jullie hebben een ontzettend grote bijdrage geleverd aan al het microscopische werk dat ik op de afdeling heb uitgevoerd. Heel erg bedankt voor de professionele en altijd weer snelle produktie van de prachtige (digitale) foto's waarmee ik iederekeer weer kwam aanzetten. Natuurlijk was er tijdens het harde werken ook altijd even tijd voor gezelligheid! De sprinkhanenverzorgers **Henk Schriek**, **Job Jansen** en **Co Rootselaar**, bedankt voor het verzorgen van al die sprinkhanen in aparte kooitjes die allemaal een speciale behandeling nodig hadden. De studenten die ik niet persoonlijk heb begeleid, maar met wie ik wel veel "gekkegeit" heb uitgehaald: **Olaf Welting**, **Maria Boersma**, **Nadine Pouw**, **Dennis van de Wijngaart**, **Jolanda Snapper**, **Lonneke Schuurmans**, **Nathalie Fu**, **Tessa Wijnhoven**, **Antoine Demorrée** en **Sjoerd Luiten**.

Een aantal mensen van buiten de Universiteit wil ik ook bedanken. Niet voor de wetenschappelijke bijdrage, maar wel voor de motivatie en minstens zo belangrijke ontspanning buiten het werk. Mijn ouders, **Frank** en **Ine**, dank ik voor het mogelijk maken van het hele opleidingstraject dat heeft geleid tot de totstandkoming van dit boekje. Het mag duidelijk zijn dat ik zonder jullie nooit zo ver was gekomen. Als dank hiervoor is het kroontje op mijn werk (dit proefschrift) dan ook aan jullie opgedragen.

Martin Bonke, zelfs honderden kilometers verwijderd ben je een gigantische steun in mijn rug geweest en gebleven. Het zal ons weinig moeite kosten om dat zo te houden. En het feit dat je nog steeds geen 180 hebt gegooid in Mick O'Connell's is naar mijn idee reden genoeg om terug te blijven komen naar Nederland. De wetenschappelijke "180" heb je al waargemaakt met je Nature publicatie, dus die promotie van jou moet ook geen probleem zijn.

Jon Sanz en **Carine Stevens**, jullie dank ik voor het bereiken van de top zowel binnen als buiten de klimhal. Dit boekje geeft aan dat ik een prachtig wetenschappelijk (voor)uitzicht heb bereikt, dus ik zou zeggen "BLOCK!"

Lieve **Wietske Harts**, Hier istie dan. Het stapeltje papier waarvoor ik mezelf maanden heb opgesloten in dat muffe werkkamertje met als enige lichtbron de monitor van mijn computer. Het is niet altijd even makkelijk geweest, maar men zegt dat dat er nu eenmaal bijhoort. Je hebt een onbeschrijflijk grote bijdrage geleverd aan mijn promotie die ik onmogelijk in dit boekje kan verwoorden zonder het totaal aantal pagina's te verdubbelen. Ik houd het kort en krachtig met de drie belangrijkste sleutelwoorden: heel erg bedankt voor de *steun*, *ontspanning* en vooral de *liefde* die je me hebt gegeven.



Bungee jump vanaf de Kawarau Bridge (hoogte: 43 meter) inclusief head-dip (kopje onder in de Kawarau rivier).

The original bungee site A. J. Hacket, Queenstown, New Zealand.

CURRICULUM VITAE

Dennis van Hoof was born on September 10th, 1976, in Maarssen. After obtaining his VWO certificate at College "De Klop" in Utrecht in 1994, he studied Biology at Utrecht University. As an undergraduate student, he characterized several *Arabidopsis thaliana* root mutants in the Department of Molecular Genetics, Faculty of Biology. In a second research project, he investigated candidate genes of *Solanum tuberosum* that confer resistance to *Globodera pallida* in the Department of Molecular Plant Physiology of CPRO-DLO in Wageningen. To finish his graduate studies, he wrote a thesis about the specificity of serine proteinase inhibitors (serpins) at the Department of Biochemical Physiology at Utrecht University.

He graduated in May 1999, and in December of that year he started the NWO-subsidized research project on receptor-mediated endocytosis of insect lipoprotein in the Department of Biochemical Physiology, Utrecht University under supervision of Prof. Dr. D.J. van der Horst and Dr. K.W. Rodenburg. The results of that research are described in this thesis. During this period, he also contributed to the education of undergraduate students in Biology and Biomedical Sciences, and he was a member of the Institute of Biomembranes.

Dennis van Hoof werd geboren op 10 september 1976 te Maarssen. Na het behalen van zijn VWO diploma aan College "De Klop" te Utrecht in 1994 studeerde hij Biologie aan de Universiteit Utrecht. Tijdens deze studie deed hij in zijn eerste stage onderzoek naar de ontwikkeling van verschillende *Arabidopsis thaliana* (zandraket) wortelmutanten bij de leerstoelgroep Moleculaire Genetica. Voor een tweede stage deed hij onderzoek naar kandidaat-genen die de *Solanum tuberosum* (aardappelplant) resistentie bieden tegen *Globodera pallida* (cystenaaltje) bij de afdeling Moleculaire Plantenfysiologie van CPRO-DLO te Wageningen. Zijn doctoraalscriptie over de specificiteit van serine proteïnase inhibitors (serpins) schreef hij bij de leerstoelgroep Stofwisselingsfysiologie van de Universiteit Utrecht.

Na het behalen van zijn doctoraal examen in mei 1999 begon hij in december van dat jaar als assistent in opleiding (AiO) aan een door NWO gesubsidieerd onderzoeksproject bij de leerstoelgroep Stofwisselingsfysiologie van de Universiteit Utrecht onder supervisie van Prof. Dr. D.J. van der Horst en Dr. K.W. Rodenburg. Hierbij werd onderzoek gedaan naar de receptor-gemedieerde endocytose van insecten-lipoproteïne. De resultaten van dat onderzoek worden beschreven in dit proefschrift. Tevens leverde hij een bijdrage aan het onderwijs aan studenten Biologie en Biomedische Wetenschappen binnen de Universiteit Utrecht, en participeerde hij in het Instituut voor Biomembranen.